

CERTIFICATE OF MAILING
37 C.F.R. § 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date below:

July 12, 2007
Date

Sharon V. Hart

Sharon V. Hart

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Philip E. Thorpe and Sophia Ran (As Amended)

Serial No.: 10/621,269

Filed: July 15, 2003

For: Selected Antibody Compositions for Binding to Aminophospholipids (As Amended)

Group Art Unit: 1642

Examiner: Goddard, L.

Atty. Dkt. No.: 4001.003000

**SECOND DECLARATION OF
PHILIP E. THORPE UNDER 37 C.F.R. § 1.132**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, PHILIP E. THORPE, HEREBY DECLARE AS FOLLOWS:

1. I am a co-inventor of the subject matter disclosed and claimed in the captioned patent application.
2. I am Professor of Pharmacology and hold the Serena S. Simmons Distinguished Chair in Cancer Immunopharmacology at the Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, U.S.A. A copy of my *Curriculum Vitae* is attached as Exhibit A.

3. I have reviewed the captioned patent application again. I understand the claims in the captioned patent application to be drawn to purified antibodies, or antigen-binding fragments thereof, which bind to phosphatidylserine (PS), preferably in an ELISA recited in the claims, and have the characteristics as defined in the claims; and to compositions comprising, hybridomas producing and methods for preparing such antibodies.

4. I have reviewed the second Official Action issued by the U.S. Patent and Trademark Office (P.T.O.), the agency charged with assessing the patentability of the captioned patent application.

5. I have also reviewed again the documents filed after the first Official Action in the captioned patent application, including the portion of such documents that revised the specification of the captioned patent application.

6. In the second Official Action in the captioned patent application, I understand that the P.T.O. has questioned the earlier changes to the specification made in regard to the serum-dependence of antibodies other than the 3G4 antibody.

7. Table 2 in the specification of the captioned patent application sets forth the isotype and serum-dependence of a number of antibodies that bind to PS. Table 2 originally listed the 3G4 antibody and the 3B10 antibody as not being serum-dependent.

8. It has now been determined that binding of the 3G4 antibody to PS is serum-dependent. This is shown in two publications on which I am the senior author: Ran *et al.*, *Clin. Cancer Res.*, 11:1551-1562, 2005 and Luster *et al.*, *J. Biol. Chem.*, 281(40):29863-29871, 2006. I understand that copies of Ran *et al.*, 2005 and Luster *et al.*, 2006 were provided to the P.T.O. in the captioned patent application as part of an Information Disclosure Statement (IDS), and that these publications have also been cited in the third Official Action. The Luster paper available when in press as www.jbc.org/cgi/doi/10.1074/jbc.M605252200:1-20, 2006 has subsequently been published as Luster *et al.*, *J. Biol. Chem.*, 281(40):29863-29871, 2006, a copy of which is attached as **Exhibit C**.

9. I am providing the present Declaration and the attached evidence to present experimental support demonstrating that binding of the 3B10 antibody to PS is also serum-dependent.

10. Evidence of the fact that binding of the 3B10 antibody to PS is serum-dependent is presented in **Exhibit B**, which shows the results of ELISA assays conducted in the presence and absence of serum.

11. The data of **Exhibit B** were generated from an ELISA conducted in accordance with Example IV of the specification, but adapted to more particularly compare binding to PS in the presence and absence of serum. The 3B10 antibody was purified using FPLC. Binding of antibody to PS in the presence of serum was assayed using fetal bovine serum, whereas binding of antibody to PS in the absence of serum was assayed using ovalbumin, *i.e.*, an alternative to serum to provide the "blocking" function and prevent non-specific binding to the ELISA plate.

12. Briefly, PS was diluted to a concentration of 5 μ g/mL in hexane, dried on a 96-well ELISA plate (50 μ l to each well) and evaporated at room temperature. The plate was blocked either with 200 μ l of 1% ovalbumin in phosphate buffered saline, OVA/PBS (1%OVA), or 200 μ l of 10% fetal bovine serum in phosphate buffered saline, FBS/PBS (10%FBS) for 1-2 hours at room temperature. The plates were washed three times with PBS. The 3B10 antibody or a positive control antibody was diluted in 1% OVA/PBS and added to the plate. The positive control antibody is termed 2aG4, which is a mouse IgG_{2a} isotype of the 3G4 antibody of the captioned patent application. The starting concentration of antibody was 10 μ g/ml and serial 1:1 dilutions of the 10 μ g/mL antibody solutions were made in 1% OVA/PBS. The plates were sealed with plastic film, incubated for 1 hour at room temperature and washed three times with PBS. A 1:4000 dilution of goat anti-mouse antibody conjugated to horseradish peroxidase was prepared in 1% OVA/PBS and 100 μ l added to each well. The plates were sealed with adhesive film, incubated for 1 hour at room temperature and washed three times with PBS. 100 μ l peroxidase substrate solution, OPD, was added to each well of the plate. The wells were incubated for 5 minutes, the color reaction was stopped using 0.18 M H₂SO₄ and absorbance at 490 nm was measured using a plate reader.

13. **Exhibit B** shows the results of the ELISAs in the presence and absence of serum. As shown in **Exhibit B**, the 3B10 antibody binds to PS in the presence of fetal bovine serum (■), in contrast to the lack of meaningful binding in the presence of ovalbumin (□). The positive control antibody, 2aG4 (a mouse IgG_{2a} isotype of the 3G4 antibody), binds strongly to PS in the presence of fetal bovine serum (▲), in contrast to the lack of binding in the presence of ovalbumin (Δ). The data in **Exhibit B** therefore show that binding of the 3B10 antibody to PS is serum-dependent.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued thereon.

July 9, 2007

Date

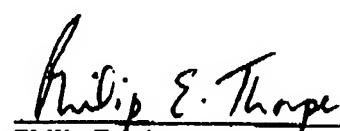

Philip E. Thorpe
Philip E. Thorpe, Ph.D.

EXHIBIT A

Curriculum Vitae - EXHIBIT A

Name: Philip Edward THORPE

Place of Birth: Smethwick, Worcestershire, U.K.

Nationality: British (with U.S. Permanent Residency)

Home Address: 5510 Morningside Drive
Dallas, TX 75206

Social Security #: 452-99-7852

EDUCATION:

1962-1969 Moseley Grammar School, Birmingham B17, U.K.

1969-1972 University of Liverpool, U.K.

Academic Qualifications: First Class B.Sc (Hons)
Degree in Pharmacology
(Summa cum Laude)

Postgraduate Education:

1972-1975 Medical Research Council Scholarship
Division of Surgical Sciences
Clinical Research Centre
London, U.K.
Ph.D. supervisors: Sir Peter Medawer, Dr. Stella Knight

POSTDOCTORAL EMPLOYMENT:

1975-1981 Medical Research Council Fellow
Division of Biology
Chester Beatty Research Institute
Institute of Cancer Research
Royal Cancer Hospital
Fulham Road
Chelsea
London SW3 6JB, U.K.

1981 - 1991 Director, Drug Targeting Laboratory
Imperial Cancer Research Fund
Lincoln's Inn Fields
London WC2A 3PX, U.K.

1991 - 1998 Professor of Pharmacology
Serena Simmons Distinguished Chair in Cancer Immunopharmacology

Department of Pharmacology and
Hamon Center for Therapeutic Oncology Research
University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75235-8593

1998 - 1999 Director of Oncology Research
 Associate Director of the Center for Molecular Medicine
 Maine Medical Center Research Institute
 125 John Roberts Road, Suite #5
 South Portland, Maine 04106

1999 – present Professor of Pharmacology
Serena Simmons Distinguished Chair in Cancer Immunopharmacology
Simmons Comprehensive Cancer Center and Hamon Center for
Therapeutic Oncology Research
University of Texas Southwestern Medical School
NC7.340
2201 Inwood Rd.
Dallas, TX 75235-8794

Telephone: 214 648-1268 or 214 648-1499
Fax: 214 648-1613
philip.thorpe@utsouthwestern.edu

UT SOUTHWESTERN GRADUATE PROGRAM APPOINTMENTS:

Cell Regulation Graduate Program, UTSW Immunology Graduate Program, UTSW

GRADUATE SCHOOL TEACHING (annual):

Mechanisms of Drug Action Course (Director)
Medical Pharmacology Course
Human Biology and Disease Course
Cancer Biology Course
Physician's Assistant Course

UT SOUTHWESTERN COMMITTEES

Promotion and Tenure Committee, 1997-98
Graduate Admissions Committee, 1994-96
Clinical Research Scientific Review And Monitoring Committee, 1997-8
Department of Cell and Molecular Biology Review Committee, 1997-8
Radioactive Drug Research Committee, 2003-present
American Cancer Society Institutional Review Group, 2004-present

PROFESSIONAL SOCIETIES:

American Association for Cancer Research
American Association for Immunology
American Society for Pharmacology and Experimental Therapeutics
North American Vascular Biology Organization
Sigma Xi
Society for Biological Therapy

SCIENTIFIC ADVISORY BOARDS:

Scientific Advisory Board, Cytopharm Inc., Munich, Germany, 1990-1996
Scientific Advisory Board, Texcellon Inc., Dallas, TX, 1990-1993
Scientific Advisory Board, Peregrine Pharmaceuticals, Inc., Princeton, NJ, 1993-1997
Scientific Advisory Board, Repair, Inc., Portland, ME, 1998-2000
Scientific Advisory Board, Peregrine Pharmaceuticals, Inc., Tustin, CA, 1997-present
Scientific Advisory Board, Arcus Therapeutics, Inc., Boston, MA, 2000-2002
Founding Scientist, Peregrine Pharmaceuticals, Inc., Tustin, CA

EDITORIAL BOARDS:

IRCS Journal of International Research Communication, 1974-1984
Advanced Drug Delivery Research Reviews, 1985-1992
Antibody, Immunoconjugates and Radiopharmaceuticals, 1987-1995
Bioconjugate Chemistry, 1989-present
Journal of Drug Targeting, 1992-2000
Therapeutic Immunology, 1992-present
Angiogenesis, 1997-present
Cancer Biotherapy and Radiopharmaceuticals, 2004-present

INTERNATIONAL CONFERENCES ORGANIZED:

Co-organizer (with Dr. G. Gregoriadis), NATO meeting on Receptor Mediated Targeting of Drugs, Greece, 1983
Vice Chairman, Gordon Research Conference on Drug Carriers in Medicine and Biology, Ventura, CA 1996
Chairman (with Dr. Ruth Duncan), Gordon Research Conference on Drug Carriers in Medicine and Biology, Ventura, CA, 1998
Chairman, 1st International Symposium on Vascular Targeting, Boston, MA, 2002
Chairman, 2nd International Symposium on Vascular Targeting, Miami, FL, 2004

INVESTIGATIONAL NEW DRUG (IND) APPLICATIONS

RFB4-SMPT-dgA for Treatment of B-lymphoma (with Dr. E. Vitetta), 1989
RFT5-SMPT-dgA for Treatment of Hodgkin's Disease (with Dr. E. Vitetta), 1992
Tarvacin™ for Treatment for Hepatitis C virus (with Peregrine Pharmaceuticals, Inc.), 2005

HONORS AND AWARDS:

Pierce Immunotoxin Award, 1988

The State of Texas House of Representatives Resolution recognizing his contribution to cancer research, 1997.

American Cancer Society 'Award of Excellence', 1999

GRANTS (since 1991):

Past

Vascular Targeting: A New Approach to the Therapy of Solid Tumors; Dallas Biomedical Corporation; \$161,832 direct for the period of October 1, 1991-December 31, 1992.

Heparin-Steroid Conjugates: A New Class of Angiogenesis Inhibitors for Clinical Applications: Dallas Biomedical Corporation; \$134,613 direct for the period of October 1, 1991 to December 31, 1992.

Vascular Targeting: A New Approach to the Therapy of Solid Tumors; Elsa U. Pardee Foundation; \$64,843 for the period of May 1, 1992-April 30, 1993.

Vascular Targeting Program; Dallas Biomedical Corporation; \$119,123 direct for the period of January 1, 1993-June 30, 1993.

Recombinant Antibodies for Targeting the Vasculature of Solid Tumors; Elsa U. Pardee Foundation; \$128,658 direct for the period of June 1, 1993-May 31, 1995.

New Angiogenesis Inhibitors for the Therapy of Breast Cancer; American Cancer Society DHP-95; \$150,000 direct for the period of July 1, 1993-June 30, 1995.

Developmental project funded from Dr. John Minna's SPORE Grant 1 P50 CA709097 from the National Institutes of Health; \$20,000 direct for the period of September 1, 1996-August 30, 1997.

Holder of the Serena S. Simmons Distinguished Chair in Cancer Immunopharmacology, annual income \$60,000.

Vascular Targeting: A New Approach to the Therapy of Solid Tumors and Rheumatoid Arthritis; Anonymous Donor; \$1,666,665 direct for the period of September 1, 1992-December 31, 1997.

Mechanisms of drug action and disposition; National Institutes of Health T32-GM07062 (training grant, PI-Dr. Alfred Gilman); \$54,000 direct for the period of September 1, 1994-August 31, 1999.

Vascular Targeting Agents that Home to and Destroy or Coagulate Tumor Vasculature; Peregrine Pharmaceuticals; \$480,000 for the period of December 1, 1994-December 1, 1997.

Vascular Targeting Agents for Infarcting Lung Cancer; Advanced Research Program from the State of Texas; \$247,500 direct for the period of January 1, 1996-December 31, 1997.

Therapeutic Clotting to Destroy Solid Tumors; Advanced Technology Program from the State of Texas; \$190,579 for the period of January 1, 1998-December 31, 1999.

Angiogenesis Inhibitors for Therapy of Solid Tumors; National Institutes of Health 5-RO1-CA59569; \$781,718 direct for the period of December 15, 1993-November 30, 1999.

Collateral Tumor Targeting; Sponsored Research Agreement with Technicclone Corporation; \$1,050,000 direct for the period of April 1999-March 2001

Immunotoxins for the Treatment of Hodgkin's Disease; National Institutes of Health 5-RO1-CA54168; \$820,052 direct for the period of April 1, 1991-May 31, 2000.

Targeting the Vasculature of Solid Tumors; National Institutes of Health 1-RO1-CA74951; \$728,529 direct for the period of December 1, 1997-November 30, 2001.

Specific coagulation of tumor vasculature. Texas Technology ARP grant; \$200,000

Present

Novel anti-viral agents for treating Lassa fever. NIH, \$1,798,285, 2003-08.

Naked antibodies for treating cancer; Sponsored Research Agreement with Peregrine Pharmaceuticals, Inc., \$500,000 per year direct (since 1999)

Therapeutic clotting to destroy solid tumors; Gillson Longenbaugh Foundation, Houston, Texas; \$50,000 per year

Anti-angiogenic drugs for childhood brain cancer. Chesler Foundation, \$10,000 per year.

VEGF-rGel for targeting the vasculature of breast cancer (M.Rosenblum, P.I). Dept. of Defense. \$43,000 per year for 2002-5.

Simmons Foundation, Serena S. Simmons Distinguished Chair in Cancer Immunopharmacology, \$86,000 per year.

Vascular Targeting Antibodies for Improving Chemotherapy of Prostate Cancer (P. Thorpe, PI). Department of Defense; \$210,000 per year

Synergy between anti-phosphatidylserine monoclonal antibody, 3G4 and docetaxel for treatment of breast cancer (X. Huang, PI; P. Thorpe, Co-PI), Susan Komen Foundation for Basic, Clinical and Translational Breast Cancer Research; \$200,000 per year

A strategy for enhancing the effect of radiation in the treatment of breast cancer (T. Luster, Fellowship). American Cancer Society; \$80,000 per year

PATENTS

Issued

1. Heterobifunctional linking agents derived from N-succinimido-dithio-alpha methyl-methylene-benzoates (Inventor: P. Thorpe)
U.S. Patent No. 4,880,935
2. Purification of A-chain immunotoxins (Inventor: P. Thorpe)
U.K. Patent No. 43606 P3474
3. Methods and compositions for the treatment of Hodgkin's disease (Inventors: P. Thorpe and A. Engert)
U.S. Patent No. 5,165,923
4. Preparation and use of steroid-polyanionic polymer-based conjugates targeted to vascular endothelial cells (Inventor: P. Thorpe)
U.S. Patent No. 5,474,765
U.S. Patent No. 5,762,918
5. Methods and compositions for targeting the vasculature of solid tumors (Inventors: P. Thorpe and F. Burrows)
U.S. Patent No. 6,004,554
U.S. Patent No. 5,965,132
U.S. Patent No. 5,855,866
U.S. Patent No. 5,776,427
U.S. Patent No. 5,863,538
U.S. Patent No. 6,051,230
U.S. Patent No. 6,261,535
European Patent No. 0 627 940 (17 countries, including France, Germany, U.K.)
6. Antibodies that bind to endoglin (Inventors: P. Thorpe and F. Burrows)
U.S. Patent No. 5,660,827
7. VEGF-Gelonin for targeting the vasculature of solid tumors (Inventor: P. Thorpe)
U.S. Patent No. 6,451,312
8. Methods and compositions for the coagulation of tumor vasculature (Inventors: P. Thorpe and T. Edgington)
U.S. Patent No. 6,093,399
U.S. Patent No. 6,004,555
U.S. Patent No. 5,877,289
U.S. Patent No. 6,036,955
U.S. Patent No. 6,749,853
European Patent No. 0 771 216 (16 countries, including France, Germany, U.K.)
Australian Patent No. 702250
New Zealand Patent No. 288883
Hungarian Patent No. 220347
Singapore Patent No. 35823
Mexican Patent No. 212,225

9. Tissue Factor methods, compositions and combination for coagulation and tumor treatment (Inventors: P. Thorpe, S. King and B. Gao)
U.S. Patent No. 6,156,321
U.S. Patent No. 6,132,729
U.S. Patent No. 6,132,730
European Patent No. 0 988 056 (15 countries, including France, Germany, U.K.)
Australian Patent No. 735187
New Zealand Patent No. 336720
Singapore Patent No. 66589
10. Cancer treatment methods using antibodies to aminophospholipids (Inventors: P. Thorpe, S. Ran)
U.S. Patent No. 6,406,693
Australian Patent No. 771224
New Zealand Patent No. 508950
11. Cancer treatment methods using therapeutic conjugates that bind to aminophospholipids (Inventors: P. Thorpe, S. Ran, R. Brekken)
U.S. Patent No. 6,312,694
European Patent No. 1 098 665 (15 countries, including France, Germany, U.K.)
Australian Patent No. 750414
Singapore Patent No. 78111
New Zealand Patent No. 508873
12. Antibody and antibody conjugate compositions and kits for selectively inhibiting VEGF (Inventors: P. Thorpe, R. Brekken)
U.S. Patent No. 6,342,219
U.S. Patent No. 6,342,221
U.S. Patent No. 6,416,758
U.S. Patent No. 6,524,583
U.S. Patent No. 6,676,941
U.S. Patent No. 6,703,020
Australian Patent No. 774287
Australian Patent No. 763954
European Patent No. 1 179 541
South African Patent No. 2001/8612
South African Patent No. 2001/8285

Pending

44 pending regular U.S. patent applications and 186 pending international patent applications directed to compositions and methods for the diagnosis and treatment of cancer and viral infections

PUBLICATIONS:(Total = 173 plus 1 submitted)

1. Thorpe, P. E. and Knight, S. C. (1974) Microplate culture of mouse lymph node cells. I. Quantitation of responses to allogeneic lymphocytes and phytomitogens. *J. Immunol. Methods* **5**: 387-404.
2. Thorpe P. E., Knight, S. C. and Farrant, J. (1976) Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. *Cryobiology* **13**: 126-138.
3. Thorpe, P. E., Ross, W. C. J., Cumber, A. J., Hinson, C. A., Edwards, D. C. and Davies, A. J. S. (1978) Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to anti-lymphocytic globulin. *Nature* **271**: 752-754.
4. Ross, W. C. J., Thorpe, P. E., Cumber, A. J., Edwards, D. C., Hinson, C. A., and Davies, A. J. S. (1980) Increased toxicity of diphtheria toxin for human lymphoblastoid cells following covalent linkage to anti-(human lymphocyte) globulin or its $F(ab^1)_2$ fragment. *Eur. J. Biochem.* **104**: 381-390.
5. Davies, A. J. S., Edwards, D. C. and Thorpe, P. E. (1981) Introduction to a symposium on new trends in human immunology and cancer immunotherapy. In 'New Trends in Human Immunology and Cancer Immunotherapy'. pp 1-7.
6. Thorpe, P. E., Cumber, A. J., Williams, N., Edwards, D. C., Ross, W. C. J. and Davies, A. J. S. (1981) Abrogation of the non-specific toxicity of abrin conjugated to anti-lymphocyte globulin. *Clin. Exp. Immunol.* **43**: 195-200.
7. Thorpe, P. E., Brown, A. N. F., Ross, W. C. J., Cumber, A. J., Detre, S. I., Edwards, D. C., Davies, A. J. S. and Stirpe, F. (1981) Cytotoxicity acquired by conjugation of an anti-Thy 1.1 monoclonal antibody and the ribosome-inactivating protein, gelonin. *Eur. J. Biochem.* **116**: 447-454.
8. Edwards, D. C. and Thorpe, P. E. (1981) Targeting toxins - the retiarian approach to chemotherapy. *Trends in Biochemical Sciences*, 313-316.
9. Edwards, D. C., Smith, A., Ross, W. C. J., Cumber, A. J., Thorpe, P. E. and Davies, A. J. S. (1981) The effect of abrin, anti-lymphocyte globulin and their conjugates on the immune response of mice to sheep red blood cells. *Experientia* **37**: 256-257.
10. Skilleter, D. N., Paine, A. J. and Thorpe, P. E. (1981) Selective direction of ricin to hepatic parenchymal cells. *Biochem. Soc. Transactions* **10**: 122-123.
11. Thorpe, P. E., Cumber, A. J., Davies, A. J. S., Edwards, D. C., Ross, W. C. J. and Smith, A. (1982) The immunosuppressive effects of anti-Thy 1.1 $F(ab^1)_2$ conjugated to abrin. In 'Antibodies as Carriers of Anticancer Drugs or Toxins: Quo Vadis?' (F. K. Jansen and R. Roncucci, eds.) SANOFI, Montpellier, France, pp. 134-135.
12. Thorpe, P. E., Brown, A., Cumber, A. J., Davies, A. J. S., Edwards, D. C., Ross, W. C. J. and Stirpe, F. (1982) Selective cytotoxicity with a conjugate of anti-Thy 1.1 antibody and gelonin.

In 'Antibodies as Carriers of Anticancer Drugs or Toxins: Quo Vadis?' (F. K. Jansen and R. Roncucci, eds.) SANOFI, Montpellier, France, pp. 123-124.

13. Edwards, D. C., Ross, W. C. J., Cumber, A. J., McIntosh, D., Smith, A., Thorpe, P. E., Brown, A., Williams, R. H. and Davies, A. J. S. (1982) A comparison of the in vitro and in vivo activities of conjugates of anti-mouse lymphocyte globulin and abrin. *Biochim. Biophys. Acta* **71**: 272-277.
14. Edwards, D. C., Thorpe, P. E. and Davies, A. J. S. (1982) Antibody-toxin conjugates as potential therapeutic agents. In 'Targeting of Drugs' (G. Gregoriadis, J. Senior, and A. Trouet, eds.) Plenum Press, N. Y. and London, pp. 83-96.
15. Thorpe, P. E., Ross, W. C. J. (1982) The preparation and cytotoxic properties of antibody-toxin conjugates. *Immunol. Rev.* **62**: 119-158.
16. Thorpe, P. E., Edwards, D. C., Davies, A. J. S., Ross, W. C. J. (1982) Monoclonal antibody-toxin conjugates: aiming the magic bullet. In 'Monoclonal Antibodies in Clinical Medicine' (A. McMichael and J. Fabre, eds.) Acad. Press, London, pp. 167-201.
17. Thorpe, P. E., Mason, D. W., Brown, A. N. F., Simmonds, S. J., Ross, W. C. J., Cumber, A. J. and Forrester, J. A. (1982) Selective killing of malignant cells in a leukaemic rat bone marrow using an antibody-ricin conjugate. *Nature* **297**: 594-596.
18. Mason, D. W., Thorpe, P. E., Ross, W. C. J. (1982) Elimination of leukaemic cells from rodent bone marrow in vitro with antibody-ricin conjugates: implications for autologous marrow transplantation in man. *Cancer Surveys* **1**: 389-415.
19. Davies, A. J. S., Jansen, F. K., Olsnes, S., Thorpe, P. E., Wofsy, L. and Edwards, D. C. (1982) Antibodies as toxin carriers in cancer immunotherapy. In 'Current Chemotherapy and Immunotherapy' (Proceedings of the 12th Int. Congress of Chemotherapy, Vol. 2) (Periti, P. and Grassi, G. G. Eds.) pp. 1141-1143.
20. Thorpe, P. E., Brown, A., Foxwell, B. and Myers, C. (1983) Blockade of the galactose-binding site of ricin by its linkage to antibody. In 'Monoclonal Antibodies and Cancer' (B. D. Boss, R. Langman, I. Trowbridge and R. Dulbecco, eds.) Acad. Press (London) Ltd., pp. 117-124.
21. Vodinelich, L., Myers., C., Sutherland, R., Thorpe, P. E. and Greaves, M. F. (1983) WT1: a monoclonal antibody in T-cell acute lymphoblastic leukemia. *Leukemia Reviews International* **1**, 263.
22. Thorpe, P. E., Detre, S. I., Mason, D. W., Cumber, A. J. and Ross, W. C. J. (1983) Monoclonal antibody therapy: 'model' experiments with toxin conjugated antibodies in mice and rats. *Haematology and Blood Transfusion* **28**: 107-111.
23. Rennie, D. P., McGregor, A. M., Wright, J., Weetman, A. P., Hall, R. and Thorpe, P. E. (1983) An immunotoxin of ricin A chain conjugated to thyroglobulin selectively suppresses the antithyroglobulin autoantibody response. *Lancet* **ii**, 1338-1340.
24. Thorpe, P. E., Ross, W. C. J., Brown, A. N. F., Myers, C. D., Cumber, A. J., Foxwell, B. M. J. and Forrester, J. A. (1984) Blockade of the galactose-binding sites of ricin by its linkage to

- antibody: specific cytotoxic effects of the conjugates. *Eur. J. Biochem.* **140**: 63-71.
25. Sikora, K., Smedley, H. and Thorpe, P. E. (1984) Tumor Imaging and Drug Targeting. *Brit. Med. Bull.* **40**: 233-239.
26. Myers, C. D., Thorpe, P. E., Ross, W. C. J., Cumber, A. J., Katz, F. E., Tax, W., and Greaves, M. F. (1984) An immunotoxin with therapeutic potential in T cell leukemia: WT1-ricin A. *Blood* **63**: 1178-1185.
27. Foxwell, B. M. J., Ross, W. C. J. and Thorpe, P. E. (1984) Antibody-ricin conjugates: a method of linkage which blocks the galactose binding site of ricin. *Behring Inst. Mitt.*, **74**: 101-107.
28. Thorpe, P. E. (1984) Antibody-toxin conjugates as anti-cancer agents. In 'Cancer Chemotherapy and Selective Drug Development' (Harrap, K. R., Davies, W. and Calvert, A. H. eds.) Martinus-Nijhoff Publishing Co., Boston, The Hague, Dordrecht and Lancaster, pp. 263-267.
29. Paraskeva, C., Buckle, B. G. and Thorpe, P. E. (1985) Selective killing of contaminating human fibroblasts in epithelial cultures derived from colorectal tumours using an anti-Thy-1 antibody-ricin conjugate. *Br. J. Cancer* **51**: 131-134.
30. Foxwell, B. M. J., Detre, S. I., Donovan, T. A. and Thorpe, P. E. (1985) The use of anti-ricin antibodies to protect mice intoxicated with ricin. *Toxicology* **34**: 79-88.
31. Cumber, A. J., Forrester, J. A., Foxwell, B. M. J., Ross, W. C. J. and Thorpe, P. E. (1985) The preparation of antibody-toxin conjugates. *Methods in Enzymology* **112**: 207-224.
32. Thorpe, P. E., Detre, S. I., Foxwell, B. M. J., Brown, A. N. F., Skilleter, D. N., Wilson, G., Forrester J. A. and Stirpe, F. (1985) Modification of the carbohydrate in ricin with metaperiodate-cyanoborohydride mixtures: effects on toxicity and *in vivo* distribution. *Eur. J. Biochem.* **147**: 197-206.
33. McIntosh, D. and Thorpe, P. (1985) Role of the B-chain in the cytotoxic action of antibody-ricin and antibody-abrin conjugates. In 'Receptor-Mediated Targeting of Drugs' (Gregoriadis, G., Poste, G., Senior, J. and Trouet, A. eds.) NATO ASI Series A, **82**: 105-118.
34. Foxwell, B. M. J., Donovan, T. A., Thorpe, P. E. and Wilson, G. (1985) The removal of carbohydrates from ricin with endoglycosidases H, F, and D and β -mannosidase. *Biochim. Biophys. Acta* **840**: 193-203.
35. Lord, J. M., Roberts, L. M., Thorpe, P. E. and Vitetta, E. S. (1985) Immunotoxins. *Trends in Biotechnology* **3**: 175-180.
36. Thorpe, P. E., Brown, A. N. F., Bremner, J. A. G., Foxwell, B. M. J. and Stirpe, F. (1985) An immunotoxin composed of monoclonal anti-Thy 1.1 antibody and a ribosome-inactivating protein from Saponaria officinalis: Potent antitumor effects *in vitro* and *in vivo*. *J. Natl. Cancer Inst. (USA)* **75**: 151-159.
37. Vitetta, E. S. and Thorpe, P. E. (1985) Immunotoxins containing ricin A or B chains with

modified carbohydrate residues act synergistically in killing neoplastic B cells *in vitro*. *Cancer Drug Delivery* **2**: 191-198.

38. Thorpe, P. E. (1985) Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review. In 'Monoclonal Antibodies '84: Biological and Clinical Applications'. (A. Pinchera, G. Doria, F. Dammacco and A. Bargellesi, eds.) Editrice Kurtis s.r.l., Milan, Italy. pp. 475-512.
39. Skilleter, D. N., Price, R. J., Thorpe, P. E. (1985) Modification of the carbohydrate in ricin with metaperiodate and cyanoborohydride mixtures: effect on binding, uptake and toxicity to parenchymal and non-parenchymal cells of rat liver. *Biochim. Biophys. Acta* **842**: 12-21.
40. Skilleter, D. N., Price, R. J., Thorpe, P. E. and Foxwell, B. M. J. (1985) Mannose oligosaccharides in ricin are involved in the selective uptake of the toxin by hepatic non-parenchymal cells. *Human Toxicology* **5**: 126, 1986.
41. Wawrzynczak, E. and Thorpe, P. E. (1986) Monoclonal Antibodies and Therapy. In 'Introduction to the Cellular and Molecular Biology of Cancer' (L. M. Franks and N. M. Teich, eds.) Oxford University Press, (Oxford, N. Y. & Tokyo) pp. 378-410.
42. Blakey, D. C. and Thorpe, P. E. (1986) Immunotoxins. *BioEssays* **4**: 292-297.
43. Fulton, R. J., Blakey, D. C., Knowles, P. P., Uhr, J. W., Thorpe, P. E., Vitetta, E. S. (1986) Purification of ricin A₁, A₂ and B-chains and characterization of their toxicity. *J. Biol. Chem.* **261**: 5314-5319.
44. Blakey, D. C. and Thorpe, P. E. (1986) Effect of chemical deglycosylation on the *in vivo* fate of ricin A-chain. *Cancer Drug Delivery* **3**: 189-196.
45. Wawrzynczak, E. J. and Thorpe, P. E. (1986) Complete enzymic deglycosylation of ricin B-chain. *FEBS Letters* **207**: 213-216.
46. Foxwell, B. M. J., Blakey, D. C., Brown, A. N. F., Donovan, T. A. and Thorpe, P. E. (1987) The preparation of deglycosylated ricin by recombination of glycosidase-treated A and B-chain: effects of deglycosylation on toxicity and *in vivo* distribution. *Biochim. Biophys. Acta* **923**: 59-65.
47. Thorpe, P. E. and Mason, D. W. (1987) Monoclonal antibody-toxin conjugates: theory, experimental approaches and early clinical results in marrow transplantation in man. In 'Tumour Markers' (A. S. Daar, ed.) Blackwell Scientific Publ. Ltd., (London) pp. 178-203.
48. Vitetta, E. S. and Thorpe, P. E. (1987) Immunotoxins. In 'New avenues in Developmental Cancer Chemotherapy' (Proceedings of the 8th Annual Bristol-Myers Symposium on Cancer Research'. (K. Harrap and T. A. Connors, eds.) Academic Press, N. Y. and London. pp. 265-276.
49. Knowles, P. P. and Thorpe, P. E. (1987) Purification of immunotoxins containing ricin A-chain and abrin A-chain using Blue Sepharose CL-6B. *Anal. Biochem.* **160**: 440-443.
50. Blakey, D. C., Watson, G. J., Knowles, P. P., and Thorpe, P. E. (1987) Effect of chemical

deglycosylation of ricin A-chain on the in vivo fate and cytotoxic activity of an immunotoxin composed of ricin A-chain and anti-Thy 1.1 antibody. *Cancer Res.* **47**: 947-952.

51. Blakey, D. C., Wawrzynczak, E. J., Stirpe, F. and Thorpe, P. E. (1987) Anti-tumour activity of a panel of anti-Thy 1.1 immunotoxins made with different ribosome inactivating proteins. In 'Membrane-Mediated Cytotoxicity'. UCLA Symposia on Molecular and Cellular Biology, Volume **45** (B. Bonavida and R. J. Collier, eds.) Alan R. Liss, Inc., pp. 195-202.
52. Wawrzynczak, E. J., Falasca, A., Jeffery, W. A., Watson, G. M. and Thorpe, P. E. (1987) Identification of a tyrosine residue in the saccharide binding site of ricin B-chain using N-[¹⁴C]-acetylimidazole. *FEBS Letters* **219**: 51-55.
53. O'Hare, M., Roberts, L. M., Thorpe, P. E., Watson, G. J. Prior, B. and Lord, J. M. (1987) Expression of ricin A chain in Escherichia coli. *FEBS Letters*, **216**: 73-78.
54. Stirpe, F., Derenzini, M., Barbieri, L., Farabegoli, F., Brown, A. N. F., Knowles, P. P., Thorpe, P. E. (1987) Hepatotoxicity of immunotoxins made from saporin, a ribosome-inactivating protein from Saponaria officinalis. *Virchows Arch. B Cell Path* **53**: 259-271.
55. Glennie, M. J., McBride, H. M., Worth, A. T., Stirpe, F., Thorpe, P. E. and Stevenson, G. T. (1987) Emergence of immunoglobulin variants following treatment of a B-cell leukemia with an immunotoxin composed of anti-idiotypic antibody and saporin. *J. Exp. Med.* **166**: 43-62.
56. Wawrzynczak, E. and Thorpe, P. E. (1987) Methods for preparing immunotoxins: effect of the linkage on activity and stability. In 'Immunotoxins: current methods and approaches for the targeting of toxins and other substances.' (Vogel, C. W., ed.) Oxford University Press, pp. 28-55.
57. Thorpe, P. E., Wallace, P. M., Knowles, P. P., Relf, M. G., Brown, A. N. F., Watson, G. J., Knyba, R. E. Wawrzynczak, E. J. and Blakey, D. C. (1987) New coupling agents for the synthesis of immunotoxins containing a hindered disulfide bond with improved stability in vivo. *Cancer Res.* **47**: 5924-5931.
58. Katz, F. E., Janossy, G., Cumber, A., Ross, W., Blacklock, H. A., Tax, W. and Thorpe, P. E. (1987) Elimination of T cells from human peripheral blood and bone marrow using a cocktail of three anti-T cell immunotoxins. *Br. J. Haematol.* **67**: 407-411.
59. Thorpe, P. E., Blakey, D. C., Brown, A. N. F., Knowles, P. P., Knyba, R. E., Wallace, P. M., Watson, G. J. and Wawrzynczak, E. (1987) Comparison of two anti-Thy 1.1-abrin A-chain immunotoxins prepared with different crosslinking agents: antitumor effects, in vivo fate and tumor cell mutants. *J. Natl. Cancer Inst.* **79**: 1101-1112.
60. Barnett, M.J., Rohatiner, A.Z., Kingston, J.E., Adams, K.E., Batten, E.L., Bassen, R., Thorpe, P.E., Horton, M.A., Malpas, J.S. and Lister, T.A. (1987) In vitro treatment of bone marrow from patients with T-cell acute lymphoblastic leukemia and non-Hodgkin's lymphoma using the immunotoxin WT1-ricin A. *Hematologie Und Bluttransfusion* **31**: 57-58.
61. Blakey, D.C. and Thorpe, P. E. (1988) An overview of therapy with immunotoxins containing ricin or its A-chain. *ANTIBODY, Immunoconjugates and Radiopharmaceuticals*, **1**: 1-16.

62. Blakey, D. C., Skilleter, D. N., Price, R. J. and Thorpe P. E. (1988) Uptake of native and deglycosylated ricin A-chain immunotoxins by mouse liver parenchymal and non-parenchymal cells in vitro and in vivo. *Biochim. Biophys. Acta* **968**: 172-178.
63. Till, M., May, R. D., Uhr, J. W., Thorpe, P. E. and Vitetta, E. S. (1988) An assay that predicts the ability of monoclonal antibodies to form potent ricin A chain-containing immunotoxins. *Cancer Res.* **48**: 1119-1123.
64. Blakey, D.C., Watson, G. J., Knowles, P. P. and Thorpe, P.E. (1988) The effect of chemical deglycosylation of ricin A-chain on the therapeutic potential of ricin A-chain immunotoxins. In 'Proceedings of International Symposium on Lectins and Glycoconjugates in Oncology'. Springer-Verlag Press, pp. 97-102.
65. Ghetie, M., May, R. D., Till, M., Uhr, J. W., Ghetie, V., Knowles, P. P., Relf, M., Brown, A. N., Wallace, P. M., Janossy, G., Amlot, P., Vitetta, E. S. and Thorpe, P. E. (1988) Evaluation of ricin A chain-containing immunotoxins directed against CD19 and CD22 antigens on normal and malignant human B cells as potential reagents for in vivo therapy. *Cancer Res.* **48**: 2610-2617.
66. Foxwell, B. M. J., Band, H. A., Long, J., Jeffery, W. A., Snook, D., Thorpe, P. E., Watson, G., Parker, P., Epenetos, A. A. and Creighton, A. M. (1988) Conjugation of monoclonal antibodies to a synthetic peptide substrate for protein kinase: a method for the labelling of antibodies with ^{32}P . *Br. J. Cancer* **57**: 489-493.
67. Blakey, D. C. and Thorpe, P. E. (1988) The prevention of carbohydrate-mediated clearance of ricin-containing immunotoxins by the liver. In 'Immunotoxins'. (A.E. Frankel, ed.) Martinus Nijhoff Publ., Boston pp. 457-473.
68. Wawrzynczak, E. J. and Thorpe, P. E. (1988) Effect of chemical linkage upon the stability and cytotoxic activity of A-chain immunotoxins. In 'Immunotoxins' (A. E. Frankel, ed.) Martinus Nijhoff Publ., Boston pp. 239-251.
69. Drobniowski, F. A., Thorpe, P. E., Wallace, P. M. and Wawrzynczak, E. J. (1988) The potential of membrane-acting toxins for targeted cancer therapy. In 'Targeting of Drugs: Anatomical & Physiological Considerations' (G. Gregoriadis & G. Poste eds.) Plenum Press, N. Y. & London, pp. 103-108.
70. Blakey, D. C., Wawrzynczak, E. J., Wallace, P. M. and Thorpe, P. E. (1988) Antibody-toxin conjugates: a perspective. *Progress in Allergy: Monoclonal antibodies* **45**: 50-90.
71. Stirpe, F., Wawrzynczak, E. J., Brown, A. N. F., Knyba, R. E., Watson, G. J., Barbieri, L. and Thorpe, P. E. (1988) Selective cytotoxic activity of immunotoxins composed of a monoclonal anti-Thy 1.1 antibody and the ribosome-inactivating proteins, bryodin and momordin. *Brit. J. Cancer* **58**: 558-561.
72. Wawrzynczak, E. J., Drake, A. F., Watson, G. J., Thorpe, P. E. and Vitetta, E. S. (1988) Ricin B chain-containing immunotoxins prepared with heat-denatured B chain lacking galactose-binding ability potentiate the cytotoxicity of a cell-reactive ricin A-chain immunotoxin. *Biochim. Biophys. Acta* **971**: 55-62.

73. Wawrzynczak, E. J., Drake, A. F. and Thorpe, P. E. (1988) Circular dichroism of isolated ricin A- and B-chains. *J. Biophys. Chem.* **31**: 301-305.
74. Blakey, D. C., Skilleter, D. N., Price, R. J. and Thorpe, P. E. (1988) Comparison of the pharmacokinetics and hepatotoxic effects of saporin and ricin A-chain immunotoxins. *Cancer Res.* **48**: 7072-7078.
75. Thorpe, P. E., Wallace, P. M., Knowles, P. P., Relf, M. G., Brown, A. N., Watson, G. J., Blakey, D. C. and Newell, D. R. (1988) Improved antitumor effects of immunotoxins prepared with deglycosylated ricin A-chain and hindered disulfide linkages. *Cancer Res.* **48**: 6396-6403.
76. Shen, G. L., Li, J. L., Ghetie, M. A., Ghetie, V., May, R. D., Till, M., Brown, A. N., Relf, M., Knowles, P., Uhr, J. W., Janossy, G., Amlot, P., Vitetta, E. S. and Thorpe, P. E. (1988) Evaluation of four CD22 antibodies as ricin A chain-containing immunotoxins for the *in vivo* therapy of human B cell leukemias and lymphomas. *Int. J. Cancer* **42**: 792-797.
77. Blakey, D. C. and Thorpe, P. E. (1988) Treatment of malignant disease and rheumatoid arthritis using ricin A-chain immunotoxins. *Scand. J. Rheumatol. Suppl.* **76**: 279-287.
78. Thorpe, P. E. and Blakey, D. C. (1989) Antitumor effects of immunotoxins containing ricin or its A-chain. In 'Clinical Use of Monoclonal Antibodies' (Koene, R. A. P. and Tax, W. J. M., eds.) *Actua Sandoz* **14**: 92-104.
79. Press, O. W., Martin, P. J., Thorpe, P. E. and Vitetta, E. S. (1988) Ricin A-chain containing immunotoxins directed against different epitopes on the CD2 molecule differ in their ability to kill normal and neoplastic T cells. *J. Immunol.* **141**: 4410-4417.
80. Li, J. L., Shen, G. L., Ghetie, M. A., May, R. D., Till, M., Ghetie, V., Uhr, J., Janossy, G., Thorpe, P. E., Amlot, P. and Vitetta, E. S. (1989) The epitope specificity and tissue reactivity of four murine monoclonal anti-CD22 antibodies. *Cell. Immunol.* **118**: 85-99.
81. Blakey, D. C. and Thorpe, P. E. (1989) Anti-tumour effects of monoclonal antibody-ricin conjugates *in vitro* and *in vivo*. In 'Clinical Applications of Monoclonal Antibodies' (R. Hubbard and V. Marks, eds.) Plenum Press pp. 233-244.
82. Wiley, R. G., Stirpe, F., Thorpe, P. E. and Oeltmann, T. N. (1989) Neuronotoxic effects of monoclonal anti-Thy 1 antibody (OX7) coupled to the ribosome inactivating protein, saporin, as studied by suicide transport experiments in the rat. *Brain Research* **505**: 44-54.
83. Engert, A., Burrows, F., Jung, W., Tazzari, P-L., Stein, H., Pfreundschuh, M., Diehl, V. and Thorpe, P. E. (1990) Evaluation of ricin A-chain-containing immunotoxins directed against the CD30 antigen as potential reagents for the treatment of Hodgkin's disease. *Cancer Res.* **50**: 84-88.
84. Engert, A., Martin, G., Pfreundschuh, M., Diehl, V. and Thorpe, P. E. (1990) Comparison of antitumour effects of ricin A-chain IgG and Fab' immunotoxins for the treatment of Hodgkin's disease. In 'Trends in Drug Research' (V. Claassen, Ed.) *Pharmacochemistry Library* **13**, pp. 351-361. Elsevier, Amsterdam.

85. Engert, A., Pfreundschuh, M., Diehl, V. and Thorpe, P. E. Development of ricin A-chain immunotoxins for the treatment of Hodgkin's disease and Ki-1 lymphoma. In 'Recent Advances in Chemotherapy' (D. Adam & E. Rubinstein, eds.) Proc. 16th Int. Congr. Chemother., Jerusalem, 1989, pp. 8891-8892.
86. Engert, A., Martin, G., Pfreundschuh, M., Amlot, P., Hsu, S-M., Diehl, V. and Thorpe, P. E. (1990) Antitumor effects of ricin A-chain immunotoxins prepared from intact antibodies and Fab' fragments on solid human Hodgkin's disease tumors in mice. *Cancer Res.* **50**: 84-88.
87. Wawrzynczak, E. J. and Thorpe, P. E. (1991) Monoclonal Antibodies and Therapy. In 'Introduction to the Cellular and Molecular Biology of Cancer' Second Edition. (L. M. Franks and N. M. Teich, eds.) Oxford University Press (Oxford, N.Y. and Tokyo) pp. 468-509.
88. Engert, A. and Thorpe, P. E. (1990) Antitumor effects of six ricin A-chain immunotoxins of potential use in the treatment of Hodgkin's disease. In 'Targeting of Drugs: Optimization strategies' (G. Gregoriadis, Ed.) Plenum Press, N. Y. & London, pp. 39-48.
89. Engert, A., Thorpe, P. (1990) Immunotoxine: von der Idee der "magic bullet" bis zur klinischen anwendung. *Med. Klinik* **85**: 555-560.
90. Wawrzynczak, E. J., Watson, G. J., Cumber, A. J., Henry, R. V., Parnell, G. D., Reiber, E. P. and Thorpe, P. E. (1991) Blocked and non-blocked immunotoxins against the CD4 antigen exhibit higher cytotoxic potency than a ricin A-chain immunotoxin potentiated with ricin B-chain or with a ricin B-chain immunotoxin. *Cancer Immunol. Immunother.* **32**: 289-295.
91. O'Hare, M., Brown, A. N., Hussain, K., Gebhardt, A., Watson, G., Roberts, L. M., Vitetta, E. S., Thorpe, P. E. and Lord, M. (1990) Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence. *FEBS* **274**: 200-204.
92. Vitetta, E. S. and Thorpe, P. E. (1991) Immunotoxins. In 'Biologic Therapy of Cancer: Principles and Practice'. (DeVita V., Hellman, S., Rosenberg, S. Eds.). J. B. Lippincott Co. pp. 482-495.
93. Burrows, F. J., Haskard, D. O., Hart, I. R., Poole, S., Thorpe, P. E. (1991) Influence of tumor-derived IL-1 on melanoma-endothelial cell interactions in vitro. *Cancer Res.* **51**: 4768-4775.
94. Engert, A., Martin, G., Amlot, P., Wijdenes, J., Diehl, V. and Thorpe, P. (1991) Immunotoxins constructed with CD25 monoclonal antibodies and deglycosylated ricin A-chain have potent antitumor effects against human Hodgkin cells in vitro and solid Hodgkin tumors in mice. *Int. J. Cancer* **49**: 450-456.
95. Vitetta, E. S., Uhr, J. W., Stone, M., Fay, J., May, R., Till, M., Amlot, P., Newman, J., Cunningham, D., Clark, P., Ghetie, V. and Thorpe, P. E. (1991) A Phase I immunotoxin trial in patients with B cell lymphoma. *Cancer Res.* **51**: 4052-4058.
96. Vitetta, E. S. and Thorpe, P. E. (1991) Immunotoxins containing ricin or its A-chain. *Seminars in Cell Biology*. **2**: 47-58.

97. Ghetie V., Thorpe, P., Ghetie, M-A., Knowles, P., Uhr, J. W. and Vitetta, E. S. (1991) The GLP large scale preparation of immunotoxins containing deglycosylated ricin A-chain and a hindered disulfide bond. *J. Immunol. Meth.* **142**: 223-230.
98. Engert, A., Brown, A. and Thorpe, P. (1991) Resistance of myeloid leukemia cell lines to ricin A-chain immunotoxins. *Leukemia Res.* **15**: 1079-1086.
99. Thorpe, P.E., Wallace, P.M., Knyba, R.E., Watson, G.J., Mahadevan, V.A., Land, H., Yerganian, G., and Brown, P.J. (1991) Selective killing of proliferating vascular endothelial cells by an anti-fibronectin receptor immunotoxin. *Int. J. Radiat. Biol.* **60**: 24.
100. Lord, J.M., Roberts, L.M. and Thorpe, P.E. (1992) Chimeric proteins containing ricin A chain. In 'Genetically Engineered Toxins'. (A.E. Frankel, Ed). Marcel-Dekker, N.Y., pp. 183-190.
101. Engert, A., Brown, A., Diehl, V. and Thorpe, P. (1992) Screening of 19 monoclonal antibodies for their potential as ricin A-chain immunotoxins against myeloid leukemia cell lines. *Haematology and Blood Transfus.* **34**: 373-376.
102. Burrows, F. J., Watanabe, Y., and Thorpe, P. E. (1992) A murine model for antibody-directed targeting of vascular endothelial cells in solid tumors. *Cancer Res.* **52**: 5954-5962.
103. Engert, A. and Thorpe, P. E. (1992) The development of ricin A-chain immunotoxins for clinical trials in patients with Hodgkin's disease. In 'Targeting of Drugs 3: The Challenge of Peptides & Proteins' (G. Gregoriadis, A.T. Florence and G. Poste, eds.) Plenum Press, London, pp. 9-17.
104. Engert, A., Gottstein, C., Winkler, U., Schon, G., Amlot, P., Thorpe, P. and Diehl, V. (1992) New perspectives in oncology: Is selective destruction of tumor cells with immunotoxins in Hodgkin's disease an additional therapeutic alternative? *Med. Klin.* **87**: 503-509.
105. Vitetta, E. S., Uhr, J. W. and Thorpe, P. E. (1993) Immunotoxin therapy. In 'Cancer: Principles & Practice of Oncology' (V. DeVita, S. Hellman, S. Rosenberg) J.P. Lippincott Publ. Co., Philadelphia, pp. 2624-2636.
106. Thorpe, P. E., Derbyshire, E., Andrade, S. P., Press, N., Knowles, P. P., King, S., Watson, G.J and Rao-Bette, M. (1993) Heparin-cortisol conjugates: New angiogenesis inhibitors with anti-tumor activity in mice. *Cancer Res.* **53**: 3000-3007.
107. Vitetta, E. S., Thorpe, P. E., Uhr, J. (1993) Immunotoxins: magic bullets or misguided missiles. *Trends in Pharmacological Sciences.* **14**: 148-154 and *Immunology Today* **14**: 252-259, 1993 (joint issue).
108. Burrows, F.J. and Thorpe, P.E. (1993) Eradication of large solid tumours in mice with an immunotoxin directed against tumour vasculature. *Proc. Natl. Acad. Sci. USA* **90**: 8996-9000.
109. Drobniowski, F.A., Watson, G.J., Wawrzynczak, E.J., Alouf, J.E. and Thorpe, P.E. (1993) A novel membrane-acting immunotoxin, the immunolysin, with therapeutic potential. *Biochem. Soc. Trans.* **20**: 318S.

110. Amlot, P.L., Stone, M.J., Cunningham, D., Fay, J., Newman, J., Collins, R., May, R., McCarthy, M., Richardson, J., Ghetie, V., Ramilo, O., Thorpe, P.E., Uhr, J.W., and Vitetta, E.S. (1993) A phase I study of an anti-CD22-deglycosylated ricin A chain immunotoxin in the treatment of B cell lymphomas resistant to conventional therapy. *Blood* **82**: 2624-2633.
111. Winkler, U., Gottstein, C., Schön, G., Kapp, U., Wolf, J., Hansmann, M-L., Bohlen, H., Thorpe, P., Diehl, V. and Engert, A. (1994) Successful treatment of disseminated human Hodgkin's disease in SCID mice with deglycosylated ricin A-chain immunotoxins. *Blood* **83**: 466-475.
112. Burrows, F. J. and Thorpe, P. E. (1994) Targeting the vasculature of solid tumors. *J. Controlled Release* **28**: 195-202..
113. Burrows, F.J., Overholser, J.P. and Thorpe, P.E. (1994) Potent antitumor effects of an anti-tumor endothelial cell immunotoxin in a murine vascular targeting model. *Cell. Biophysics* **24/25**: 15-25.
114. Burrows, F.J. and Thorpe, P.E. (1994) Vascular Targeting - a new approach to the therapy of solid tumors. *Pharmacology and Therapeutics* **64**: 155-174.
115. Engert, A., Gottstein, C., Diehl, V. and Thorpe, P. (1994) Experimental treatment of human Hodgkin's disease with ricin A-chain immunotoxins. *Leukemia and Lymphoma* **13**: 441-448.
116. Thorpe, P.E. and Burrows, F.B. (1995) Vascular targeting as a new approach to the therapy of solid tumors: validation in a mouse tumor model. In 'Trends and Future Perspectives in Peptide and Protein Drug Delivery' (V.H.L. Lee, M. Hashida, Y. Mizushima) Harwood Academic Publ., Switzerland, pp. 241-251.
117. Thorpe, P.E. (1995) Antibody-directed targeting of the vasculature of solid tumors. *American Society of Clinical Oncology Educational Book*, pp. 374-377.
118. Derbyshire, E.J., Burrows, F.J., King, S., and Thorpe, P.E. (1995) Immunotoxins for targeting the vasculature of solid tumors. In 'Proceedings of the 7th European Conference on Bacterial Toxins', Copenhagen, June 1995.
119. Thorpe, P.E. and Burrows, F.J. (1995) Antibody-directed targeting of the vasculature of solid tumors. *Breast Cancer Res. and Treat.* **36**(2): 237-251.
120. Derbyshire, E.J., Comin, G.A., Yang, Y-C., Overholser, J., Watkins, L. and Thorpe, P.E. (1995) Anti-tumor and anti-angiogenic effects in mice of heparin conjugated to angiostatic steroids. *Int. J. Cancer* **63**:694-701.
121. Burrows, F. J., Tazzari, P-L., Amlot, P., Gazdar, A. F., Derbyshire, E. J., King, S. W., Vitetta, E. S. and Thorpe, P. E. (1995) Up-regulation of endoglin on vascular endothelial cells in human solid tumors: Implications for diagnosis and therapy. *Clinical Cancer Res.* **1**: 1623-1634.
122. Derbyshire, E.J., Yang, Y-C., Li, S., Comin, G.A., Belloir, J. and Thorpe, P.E. (1996) Heparin-steroid conjugates lacking glucocorticoid or mineralocorticoid activities inhibit the proliferation of vascular endothelial cells. *Biochim. Biophys. Acta* **1310**: 86-96.

123. Thorpe, P.E. and Burrows, F.B. (1996) Reply to the letter of Griffioen et al. on endoglin/CD105 as a tumor endothelial treatment target. *Breast Cancer Res. and Treat.* **39**: 241-242.
124. Huang, X., Molema, G., King, S., Watkins, L., Edgington, T.S. and Thorpe, P.E. (1997) Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science* **275**: 547-550.
125. Maier, J.A.M., Delia, D., Thorpe, P.E. and Gasparini, G. (1997) *In vitro* inhibition of endothelial cell growth by the anti-angiogenic drug AGM-1470 (TNP-470) and the anti-endoglin antibody TEC-11. *Anti-Cancer Drugs* **8**: 238-244.
126. Thorpe, P.E., Wawrzynczak, E.J. and Burrows, F.J. (1997) Monoclonal Antibodies and Therapy. In "Introduction to the Cellular and Molecular Biology of Cancer", Third Edition (L.M. Franks and N.M. Teich, Eds.) Oxford Univ. Press (Oxford, N.Y. and Tokyo) pp. 352-379.
127. Derbyshire, E.J. and Thorpe, P.E. (1997) Targeting the tumour endothelium using specific antibodies. In "Tumour Angiogenesis" (R. Bicknell, C. Lewis and N. Ferrara Eds.) Oxford Univ. Press (Oxford, N.Y. and Tokyo) pp. 343-356.
128. Derbyshire, E.J., Gottstein, C. and Thorpe, P.E. (1997) Immunotoxins In "Immunochemistry: A Practical Approach Series", (A.P. Johnstone and M.W. Turner, Eds.) Oxford Univ. Press (Oxford, N.Y. and Tokyo) pp. 239-273.
129. Derbyshire, E.J. and Thorpe, P.E. (1997) Targeting the vasculature of solid tumours. *J. Controlled Release* **48/2-3**: 277-288.
130. Brekken, R.A., King, S.W., and Thorpe, P.E. (1998) Vascular endothelial growth factor as a marker of tumor endothelium. *Cancer Res.* **58**: 1952-1959.
131. Ran, S., Gao, B., Duffy, S., Watkins, L., Rote, N. and Thorpe, P.E. (1998) Infarction of solid Hodgkin tumors in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Cancer Research*, **58**: 4646-53.
132. Gao, B., Li, S., and Thorpe, P.E. (1998) A simple and rapid method for purifying the extracellular domain of human tissue factor. *Thromb. Res.*, **91**(5): 249-53.
133. Huang, X., Gottstein, C., Brekken, R.A. and Thorpe, P.E. (1998) Expression of soluble VEGF receptor 2 and characterization of its binding to VEGF by surface plasmon resonance. *Biochem. Biophys. Res. Commun.*, **252**(3): 643-8.
134. Thorpe, P.E. and Ran, S. (2000) Tumor infarction by targeting tissue factor to tumor vasculature. *Cancer J.*, **6**(suppl. 3): S237-S244.
135. Giatromanolaki, A., Koukourakis, M.J., Sivridis, E., O'Byrne, K., Cox, G., Thorpe, P.E., Brekken, R.A., Gatter, K.C., Harris, A.L. (2000) Co-expression of MUC1 glycoprotein with multiple angiogenic factors in non-small cell lung cancer suggests co-activation of angiogenic and migration pathways. *Clin. Cancer Res.*, **6**:1917-1921.
136. Koukourakis, M.I., Giatromanolaki, A., Thorpe, P.E., Brekken, R.A., Sivridis, E., Kakolyris, S.,

- Georgoulias, V., Gatter, K.C., Harris, A.L. (2000) VEGF/KDR activated microvessel density vs. CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res.*, **60**:3088-3095.
137. Feng, D., Nagy, J.A., Brekken, R.A., Pettersson, A., Manseau, E.J., Pyne, K., Mulligan, R., Brekken, R., Thorpe, P.E., Dvorak, H.F., & Dvorak, A.M. (2000) Ultrastructural localization of the Vascular Permeability Factor/Vascular Endothelial Growth Factor (VPF/VEGF) Receptor-2 (FLK-1, KDR) in normal mouse kidney and in hyperpermeable vessels induced by VPF/VEGF-secreting tumors and adenoviral vectors. *J. Histochem. Cytochem.*, **48**:545-555.
138. Brekken, R.A., Overholser, J.P., Stasny, V.A., Waltenberger, J., Minna, J.D., and Thorpe, P.E. (2000) Selective inhibition of VEGFR2 activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res.*, **60**, 5117-24.
139. Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., Hanahan, D. McMahon, G. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biol.*, **2**, 737-744.
140. Thorpe, P.E., Ran, S. 2000. Vascular targeting. In "Tumor Angiogenesis and Microcirculation", Eds., Voest, E.E., D'Amore, P.A., Marcel Dekker, Inc., NY pp 549-578.
141. Gottstein, C., Wels, W., Ober, B., and Thorpe, P.E. (2001). Generation and characterization of recombinant Vascular Targeting Agents from hybridoma cell lines. *Biotechniques* **30**, 190-199.
142. Giatromanolaki, A., Koukourakis, M.I., Sivridis, E., Thorpe, P.E., Brekken, R.A., Konstantinos, S., Fountzilas, G., Gatter K.C., and Harris, A.L. (2000). Tumor specific activation of the VEGF/KDR angiogenic pathway in a subset of locally advanced squamous cell head and neck carcinomas. *Clin. & Exp. Metastasis* **18**, 313-319.
143. Giatromanolaki, A., Sivridis, E., Athanassou, N., Zois, E., Thorpe, P.E., Brekken, R.A., Gatter, K.C., Harris, A.L., Koukourakis, I.M., and Koukourakis, M.I. (2001). The angiogenic pathway 'vascular endothelial growth factor/flk-1 (KDR)-receptor in rheumatoid arthritis and osteoarthritis. *J. Pathol.*, **194**, 101 - 108.
144. Giatromanolaki, A., Sivridis, E., Brekken, R., Thorpe, P.E., Anastasiadis, P., Gatter, K. C., Harris, A.L., Koukourakis, M. Prognostic and therapeutic implications of VEGF/flk-1 (KDR) angiogenic pathway in endometrical carcinoma. *Cancer* **92**, 2569-2577 (2001)
145. Brekken, R.A. and Thorpe, P.E. (2001). VEGF-VEGF receptor complexes as markers of tumor vascular endothelium. *J. Controlled Release* **74**, 173 - 181.
146. Guo, P., Xu, L., Yang, S.-T., Pan, S., Brekken, R., Whitaker, G.B., Nagane, M., Thorpe, P., Rosenbaum, J., Huang, H.-J. S., Cavenee, W.K., Cheng, S.-Y. (2001). Vascular endothelial growth factor isoforms display distinct activities in promoting tumor angiogenesis at different anatomic sites. *Cancer Res.* **61**, 8569-8577.
147. Veenendaal, L.M., Jin, H., Ran, S., Cheung, L., Navone, N., Marks, J.W., Thorpe, P.E., Waltenberger, J. and Rosenblum, M.G. (2002). *In vitro* and *in vivo* studies of a VEGF₁₂₁/rGelonin chimeric fusion toxin targeting the neovasculature of solid tumors. *Proc. Nat'l. Acad. Sci. USA*, **99**: 7866-7871

148. Ran, S., Downes, A., Thorpe, P.E. (2002). Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Res.* **62**, 6132-40.
149. Thorpe, P.E., and Ran, S. (2002). Mapping zip codes in tumor vasculature. *Nature Pharmacogenetics* **2**, 205-6.
150. Ran, S., Rote, N., and Thorpe, P E. (2002). Phosphatidylserine is a marker of tumor vasculature and a potential target for anti-cancer drugs. *Int. J. Rad. Onc. Res.* **54**, 1479-84.
151. Ran, S., Rosenblum, M., and Thorpe, P.E. (2002) Ligand-directed destruction of tumor vasculature. In 'Biomedical Aspects of Drug Targeting' (Eds. V.R. Muzykantov and V.P. Torchilin). Kluwer Acad. Publ. Boston, pp 229-248.
152. Zhang, W., Ran, S., Huang, X., Thorpe, P.E. (2002) A monoclonal antibody that blocks VEGF binding to VEGFR2 (KDR/Flk-1) inhibits tumor growth in an orthotopic human breast cancer model . *Angiogenesis* **5**, 35-44.
153. Mason, R.P., Ran, S., and Thorpe, P.E. (2002) Quantitative assessment of tumor oxygen dynamics: molecular imaging for prognostic radiology. *J. Cell Biochem.* **87**, S39, 45-53.
154. Brantley, D.M., Cheng, N., Thompson, E.J., Lin, Q., Brekken, R.A., Thorpe, P.E., Muraoka, R.S., Cerretti, D.P., Pozzi, A., Jackson, D., Lin, C., Chen, J. (2002) Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. *Oncogene* **21**(46):7011-26.
155. Thorpe, P.E., Chaplin, D.J. and Blakey, D.C. (2003) The First International Conference on Vasculature Targeting: Meeting Overview. *Cancer Res.* **63**, 1144-1147.
156. Ran, S., Huang, X., Downes, A. and Thorpe, P.E. (2003) Evaluation of novel anti-mouse VEGFR2 antibodies as potential anti-angiogenic or vascular targeting agents for tumor therapy. *Neoplasia* **5**(4), 297-307.
157. Ran S, Mohamedali KA, Luster T, Thorpe P, and Rosenblum MG. (2003) The vascular-ablative agent, VEGF121/rGel, inhibits pulmonary metastases of MDA-MB-231 breast tumors. *Neoplasia* **5**, 297-307.
158. Thorpe, P.E. (2004) Vascular targeting agents as cancer therapeutics. *Clin Cancer Res.* **10**(2):415-427.
159. Cantara S, Donnini S, Giochetti A, Thorpe PE, Ziche M. (2004) Exogenous BH4/Bcl-2 peptide reverts coronary endothelial cell apoptosis induced by oxidative stress. *J. Vasc. Res.* **41**(2):202-207.
160. W. Pickering, E. Gray, A. H. Goodall, S. Ran, P. E. Thorpe and T. W. Barrowcliffe. (2004) Characterization of the cell-surface procoagulant activity of T-lymphoblastoid cell lines. *J. Thromb. Haemost.* **2**(3):459-467.
161. Ebos JML, Bocci G, Man S, Thorpe PE, Hicklin DJ, Zhou D, Jia X, Kerbel RS. (2004) A

- naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. *Mol. Cancer Res.* 2(6):315-326.
162. Huang X, Bennet M, Thorpe PE. (2004) Anti-tumor effects and lack of side effects in mice of an immunotoxin directed against human and mouse prostate-specific membrane antigen. *The Prostate* 61:1-11.
 163. Castro-Rivera E, Ran S, Thorpe P, Minna JD. (2004) Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer whereas VEGF₁₆₅ antagonizes this effect. *Proc. Natl. Acad. Sci. (USA)* 101:11432-11437.
 164. Stephan S, Datta K, Wang E, Li J, Brekken RA, Parangi S, Thorpe PE and Mukhopadhyay D. (2004) Effect of rapamycin alone and in combination with anti-angiogenesis therapy in an orthotopic model of human pancreatic cancer. *Clin. Cancer Res.* 10:6993-7000.
 165. Ran S, He J, Huang X, Soares M, Scothorn D, and Thorpe PE. (2005) Anti-tumor effects of a monoclonal antibody directed against anionic phospholipids on the surface of tumor blood vessels in mice. *Clin. Cancer Res.* 11:1551-1562.
 166. Huang X, Bennet M., and Thorpe PE. (2005) A monoclonal antibody that binds anionic phospholipids on tumor blood vessels enhances the anti-tumor effect of docetaxel on human breast tumors in mice. *Cancer Res.* 65, 4409-4416.
 167. Jennewein M, Qaim SM, Hermanne A, Jahn M, Tsyganov E, Seliounine S, Slavine N, Antich PA, Kulkarni PV, Thorpe PE, Mason RP, Rosch F. (2005) A new method for radiochemical separation of arsenic from irradiated germanium oxide. *Appl. Rad. Isotopes* 63, 3:343-351.
 168. Mohamedali, K., Sweeney, P. Huang, S., Thorpe, P.E. (2005) The vascular targeting fusion toxin VEGF121/rGel inhibits the growth of orthotopic human bladder carcinoma tumors. *Neoplasia Press* 7, 10:912-920(9).
 169. Beck, A.W., Luster, T.A., Miller, A.F., Holloway, S.E., Conner, C.R., Barnett, C.C., Thorpe, P.E., Fleming, J.B., and Brekken, R.A. (2006) Combination of a monoclonal anti-phosphatidylserine antibody with gemcitabine strongly inhibits the growth and metastasis of orthotopic pancreatic tumors. *Int. J. Cancer* 118, 10:2639-2643
 170. Tsyganov, E., Anderson, J., Arbique, G., Constantinesen, A., Jennewein, M., Kulkarni, P.V., Mason R.P., McColl, R.W., Oz, O., Parkey, R.W., Richer, E., Rosch, F., Seliounine, S., Slavine, N., Srivastava, S.C., Thorpe, P.E., Ziuchko, A.I., Antiche, P.A. UTSW small animal positron emission luager. *IEEE Transactious on Nuclear Science* accepted (2006)
 171. Beck, A., Brekken, R., and Thorpe, P.E. Targeting inside-out phospholipids on tumor blood vessels in pancreatic cancer. (Ed. D. Siemann) *Kluwer Press* (in press)
 172. Wilson, J., West, D.C., Thorpe, P.E. Antibody targeting of the tumor vasculature. In "Angiogenesis", Ed., Fan, T.P., Cambridge University Press, UK (in press)
 173. Thorpe, P.E., Ran, S. Targeting VCAM-1 and E-selectin on the vessels of solid tumors.

Proceedings of the 26th International Symposium on Controlled Release of Bioactive Materials
(in press)

EXHIBIT B

EXHIBIT B
Binding of 3B10 to PS is Serum Dependent

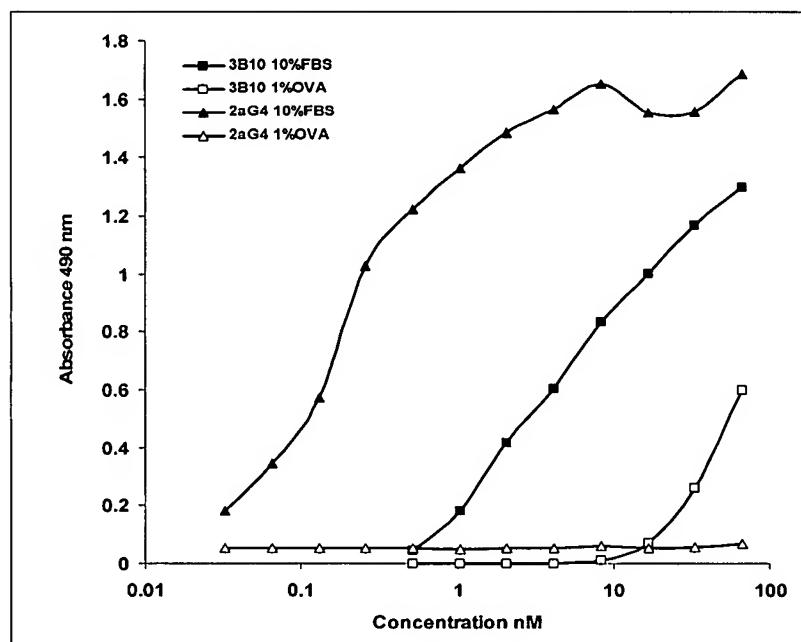


EXHIBIT C

Plasma Protein β -2-Glycoprotein 1 Mediates Interaction between the Anti-tumor Monoclonal Antibody 3G4 and Anionic Phospholipids on Endothelial Cells*

Received for publication, June 1, 2006, and in revised form, July 20, 2006. Published, JBC Papers in Press, August 10, 2006, DOI 10.1074/jbc.M605252200

Troy A. Luster[†], Jin He[‡], Xianming Huang[‡], Sourindra N. Maiti[§], Alan J. Schroit[§], Philip G. de Groot[¶], and Philip E. Thorpe^{†,1}

From the [†]Simmons Comprehensive Cancer Center, Hamon Center for Therapeutic Oncology Research, the Department of Pharmacology and the Department of Radiation Oncology, the University of Texas Southwestern Medical Center, Dallas, Texas 75390, the [‡]Department of Cancer Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and the [§]Department of Hematology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

A promising target on tumor vasculature is phosphatidylserine (PS), an anionic phospholipid that resides exclusively on the inner leaflet of the plasma membrane of resting mammalian cells. We have shown previously that PS becomes exposed on the surface of endothelial cells (EC) in solid tumors. To target PS on tumor vasculature, the murine monoclonal antibody 3G4 was developed. 3G4 localizes to tumor vasculature, inhibits tumor growth, and enhances anti-tumor chemotherapies without toxicity in mice. A chimeric version of 3G4 is in clinical trials. In this study, we investigated the basis for the interaction between 3G4 and EC with surface-exposed PS. We demonstrate that antibody binding to PS is dependent on plasma protein β -2-glycoprotein 1 (β 2GP1). β 2GP1 is a 50-kDa glycoprotein that binds weakly to anionic phospholipids under physiological conditions. We show that 3G4 enhances binding of β 2GP1 to EC induced to expose PS. We also show that divalent 3G4- β 2GP1 complexes are required for enhanced binding, since 3G4 Fab' fragments do not bind EC with exposed PS. Finally, we demonstrate that an artificial dimeric β 2GP1 construct binds to EC with exposed PS in the absence of 3G4, confirming that antibody binding is mediated by dimerization of β 2GP1. Together, these data indicate that 3G4 targets tumor EC by increasing the avidity of β 2GP1 for anionic phospholipids through formation of multivalent 3G4- β 2GP1 complexes.

We recently reported the development of a monoclonal antibody, 3G4, that targets anionic phospholipids exposed on the surface of tumor vascular endothelial cells (EC)² (1, 2). Phos-

phatidylserine (PS) is the most abundant anionic phospholipid of the plasma membrane and is considered the primary target of 3G4. It is well established that PS is actively confined to the internal leaflet of the plasma membrane under normal conditions in most cell types (3). PS asymmetry is maintained by an ATP-dependent aminophospholipid translocase that catalyzes the transport of aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane (4). Loss of PS asymmetry results from outward movement of aminophospholipids in response to increased Ca^{2+} fluxes. This leads to inhibition of the translocase (5) and/or activation of an exporter of PS that transports PS to the outer membrane surface (6). Loss of asymmetry is observed under several physiological and pathological conditions, including apoptosis (7), cell activation (8), cell injury (9), and malignant transformation (10).

Conditions in the tumor microenvironment contain a number of factors that may activate and/or injure tumor EC as follows: (a) tumor-derived interleukin-1 and tumor necrosis factor- α activate the endothelium and induce expression of cell adhesion molecules (11, 12); (b) reactive oxygen species (ROS) generated by leukocytes that adhere to the tumor endothelium (12); and (c) ROS generated by tumor cells as a by-product of metabolism (11, 13) or as a result of exposure to hypoxia followed by reoxygenation (14). In this regard, we have demonstrated that inflammatory cytokines, acidity, thrombin, hypoxia/reoxygenation, and ROS all induce PS exposure on EC *in vitro* (15). Furthermore, we have shown that 3G4 and other anti-PS monoclonal antibodies (mAbs) localize to tumor vessels following intravenous injection into mice bearing various types of primary and metastatic tumors (1, 2, 15–17). The distribution of these anti-PS mAbs was indistinguishable from that of annexin A5, which also binds PS (16). Importantly, these antibodies did not localize to vascular endothelium in non-tumor tissues. Phosphatidylserine-expressing EC in tumor vessels lack markers of apoptosis (active caspase-3, terminal deoxyribonucleotidyltransferase-mediated dUTP nick end labeling), are morphologically intact and metabolically active, and are able to transport blood and solutes (16). Thus, PS is a highly specific marker of functional, viable tumor endothelium.

* This work was supported by a Sponsored Research Agreement with Peregrine Pharmaceuticals Inc., the Gillson Longenbaugh Foundation, and a postdoctoral fellowship from the American Cancer Society (to T. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041. Tel: 214-648-1499; Fax: 214-648-1613; E-mail: philip.thorpe@utsouthwestern.edu.

² The abbreviations used are: EC, endothelial cell; ABAE, adult bovine aortic endothelial; β 2GP1, β -2-glycoprotein I; ch3G4, chimeric 3G4; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; h, human; m, mouse; LPC, lysophosphatidylcholine; mAb, monoclonal antibody; OVA, ovalbumin; PS, phosphatidylserine; ROS, reactive oxygen species; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; LPC, lysophosphatidylcholine; BSA, bovine serum albumin.

Anti-tumor mAb Requires β 2GP1 for Binding Endothelial Cells

3G4 treatment inhibits the growth of and metastatic spread of murine tumor allografts and human tumor xenografts (1), including orthotopic human breast tumors (2) and orthotopic human pancreatic tumors (18). When used in combination, 3G4 enhances the therapeutic efficacy of the chemotherapeutic drugs docetaxel and gemcitabine for treatment of breast and pancreatic tumors, respectively (2, 18). Histological evaluation of 3G4-treated tumors shows increased infiltration of host immune effector cells (primarily macrophages), along with disintegrated vessels, reduced vascularization, and increased central tumor necrosis. None of these phenomena is observed in control tumors, or normal organs taken from 3G4-treated mice. No toxicity has been observed in 3G4-treated animals as judged by extensive physiological, hematological, and histological examination, even at doses 10-fold higher than the therapeutic dose (1). A human chimeric version of 3G4 is currently in phase I clinical trials for the treatment of patients with solid tumor malignancies.

We reported previously that interaction between 3G4 and PS is dependent upon the plasma protein β -2-glycoprotein 1 (β 2GP1) (1). β 2GP1 is a member of the complement control protein family (19), consisting of five complement control protein repeats (also known as Sushi domains) in which the first four domains are regular repeats consisting of ~60 amino acids. The fifth domain differs from the other four domains as it consists of 82 amino acids, including a conserved cluster of positively charged amino acids (282–287) and a conserved hydrophobic region (amino acids 311–317) responsible for binding of β 2GP1 to anionic phospholipids (20–23). Here we demonstrate that binding of 3G4 to EC with exposed PS is β 2GP1-dependent, and we characterize the interaction required between 3G4 and β 2GP1 for binding to EC with exposed PS.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM) and trypsin/EDTA were obtained from Mediatech, Inc. (Herndon, VA). Fetal bovine serum (FBS), normal human serum, normal rat serum, and normal mouse serum were obtained from Biomedica (Foster City, CA). Fresh human plasma was obtained from Carter Blood Care (Dallas, TX). Serum-free Hybridoma Media, Synthecol NS0 supplement, L- α -phosphatidylserine (PS), bovine serum albumin (BSA), and ovalbumin from chicken egg white (OVA) were obtained from Sigma. DEAE-cellulose, heparin-Sepharose, and Hybond-P membranes were obtained from GE Healthcare. Protein C, protein S, and factor XII were obtained from Hematologic Technologies, Inc. (Essex Junction, VT). Tissue plasminogen activator and kininogen were obtained from Calbiotech (San Diego, CA). Oxidized low density lipoprotein was obtained from Intracell Resources (Frederick, MD). 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lysophosphatidylcholine (LPC)) was obtained from Avanti Polar Lipids (Alabaster, AL). Ninety six-well Immulon-1B and -2HB microtiter plates were obtained from Thermo LabSystems (Franklin, MA). Tris-HCl gradient SDS-polyacrylamide gels and an Opti-4CN substrate kit were

obtained from Bio-Rad. Eight-well glass chamber slides were obtained from BD Biosciences.

Antibodies

3G4, a mouse monoclonal antibody (mAb), was raised to bind the anionic phospholipid PS as described previously (1). 3G4 was produced originally in hybridoma supernatant but was subsequently converted to a mouse IgG2a isotype and is now produced in the NS0 mouse myeloma cell line. NS0 cells were cultured in serum-free Hybridoma Media with Synthecol NS0 supplement. A human IgG1 chimeric version of 3G4 (ch3G4) has also been generated and is produced under serum-free conditions by Peregrine Pharmaceuticals, Inc. (Tustin, CA). The mouse anti-human β 2GP1 (anti- β 2GP1) mAb was obtained from USBiological (Swampscott, MA). A hybridoma secreting C44, a colchicine-specific mouse IgG2a mAb, was obtained from the American Type Culture Collection (Manassas, VA) and used as a control for 3G4 and anti- β 2GP1. Rituximab (human IgG1 chimeric mAb) was obtained from the University of Texas Southwestern pharmacy and used as a control for ch3G4. All antibodies produced in culture supernatants were purified as described previously (24). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Preparation of Antibody Fragments

3G4 F(ab')₂ was generated by incubation with pepsin. 3G4 Fab' and control Fab' 7H11 (anti-adenovirus) were generated by reduction of F(ab')₂ counterparts. All antibody cleavage products were purified by fast protein liquid chromatography and verified by SDS-PAGE.

Purification of β 2GP1 from Human Plasma

β 2GP1 was purified from human plasma essentially as described previously (25, 26). Briefly, perchloric acid (70%) was added to pooled plasma to a final concentration of 1.57% (v/v). The precipitate was discarded, and the supernatant was adjusted to pH 7.5 with saturated Na₂CO₃, followed by extensive dialysis against 50 mM Tris, pH 8.0. This material was applied to a DEAE-cellulose column equilibrated with 50 mM Tris, pH 8.0, to remove contaminants. The DEAE column flow-through was then applied to a heparin-Sepharose affinity column equilibrated with 50 mM Tris, pH 8.0, and bound proteins were eluted using 1.0 M NaCl. Finally, the β 2GP1 preparation was dialyzed against PBS and purified further by protein A/G to remove contaminating IgG. The final preparation yielded a homogeneous 50-kDa protein, as shown by nonreduced SDS/PAGE and Coomassie staining.

Construction and Expression of Full-length and Domain-deleted Forms of Human β 2GP1 (h β 2GP1)

Strategy—To generate pure recombinant full-length and deleted forms of h β 2GP1, the yeast shuttle expression vector pPIC6 α A (Invitrogen) and host strain Mut⁺X-33 (Invitrogen) were used. The expression vector contains the 5' promoter and the 3' transcription termination sequences of the alcohol (methanol) oxidase gene (*AOX1*). The vector also has a yeast α -mating factor signal sequence downstream of the *AOX1* pro-

moter to which foreign cDNA can be fused for secretion of recombinant heterologous protein into the culture medium. Expression in *Pichia pastoris* provides glycosylation and disulfide bond formation similar to that in mammalian cells.

Generation of Expression Constructs—The following five expression constructs were made using h β 2GP1 cDNA: 1) the entire coding region of h β 2GP1 cDNA without its cognate signal peptide (domain 1–5, 5' primer used, 5'-GGAATTCTGGACGGACCTGTCCCAAGC-3'); 2) domain 1 deleted (domain 2–5, 5' primer used, 5'-GGAATTCTGTATGTC-CTTTGC-3'); 3) domain 1 and 2 deleted (domain 3–5, 5' primer used, 5'-GGAATTCTGCTCCCATCATCTGC-3'); 4) domain 1–3 deleted (domain 4–5, 5' primer used, 5'-GGAATTCTGAAATGCCATTC-3'); and 5) domain 5 only (5' primer used; 5'-GGAATTCTGCATCTTGTAAAGTAC-3'). A common 3' primer, 5'-TTCTAGATTAGCATGGCTTAC-3', was used for PCR of all fragments. PCR-amplified fragments were inserted in-frame between the EcoRI and XbaI restriction sites of pPIC α A, directly downstream from the α -mating factor signal sequence. A stop codon was introduced at the end of each fragment to prevent fusion of the recombinant proteins to a c-Myc epitope or a His tag at the C terminus. Plasmid constructs were propagated in *Escherichia coli* in the presence of 100 μ g/ml blasticidin and verified by restriction analysis and nucleotide sequencing. Recombinant proteins expressed by constructs 1–5 encoded proteins of ~36, 29, 24, 16, and 9 kDa, respectively, before glycosylation.

Transformation and Screening of Expression Clones—The recombinant plasmid constructs were linearized with restriction enzyme SacI and purified, and 10 μ g was used to transform host strain X-33 by the spheroplasts method (Invitrogen). Transformants for each of these constructs were selected on YPD (yeast extract peptone dextrose medium) plates containing 400 μ g/ml blasticidin for 4 days. Several clones for each of these constructs were restreaked on YPD plates with 400 μ g/ml blasticidin to determine the true integrants. Ten clones of each construct were then streaked on Minimal Dextrose (MD) and Minimal Methanol (MM) plates. Five clones of each construct, growing equally well on both MD and MM plates, were then grown in liquid MD and MM medium for 24, 48, 72, 96, and 120 h. Supernatants and pellets for each clone at each time point were analyzed by Western blot using anti-h β 2GP1 polyclonal antibody. Clones that showed highest expression of the protein in supernatant were further used for large scale preparation.

Large Scale Purification of the Recombinant Proteins—Recombinant proteins were produced using culture conditions recommended by Invitrogen. A starter culture of each clone was cultured in 5 ml of buffered minimal glycerol-complex medium (BMGY) at 30 °C with vigorous shaking overnight. Cells were collected, used to inoculate 25 ml of BMGY, and grown for 2 days. Cells from the 25-ml culture were then used to inoculate 1 liter of buffered minimal methanol-complex (BMMY) medium (1.0% methanol). Culture was continued for 4 days at 30 °C with vigorous shaking, and 100% methanol was added every 24 h (final concentration of 1.0%) to maintain protein expression. Culture medium was clarified by centrifugation (4000 \times g, 15 min), and supernatant was dialyzed for 2 days

at 4 °C in 50 mM Tris buffer before being applied to a DEAE-Sephadex column equilibrated with 50 mM Tris buffer. Flow-through solution was collected and applied to a heparin-Sepharose column. β 2GP1 was eluted from heparin-Sepharose column with 1 M NaCl, dialyzed against 50 mM Tris buffer, concentrated using Amicon concentrator, and analyzed by Western blot. The N terminus of each protein was sequenced to confirm cleavage of the α -factor leader sequence. Protein yields varied from 10 mg/liter (full-length β 2GP1) to 25 mg/liter (β 2GP1 domain V).

Preparation of "Nicked" h β 2GP1

Nicked h β 2GP1 was prepared from intact β 2GP1 purified from human plasma as described above. h β 2GP1 was incubated with plasmin-coated beads at 37 °C for 17 h. The beads were removed by centrifugation, and the supernatant containing the cleaved protein was recovered. Western blotting of the purified product indicated the nicked β 2GP1 preparations were plasmin-free and did not contain plasmin autoproteolytic products (no reactivity with anti-plasmin or anti-angiostatin antibodies). N-terminal sequence analysis revealed two N termini that corresponded to the N terminus of β 2GP1, and a new sequence was generated at the Lys-317/Thr-318 cleavage site.

Anti-PS ELISA

The assay was performed as described previously (1) with the following modifications. PS-coated Immunon 1B microtiter plates were blocked overnight in 1% OVA (w/v). The following day, serial 2-fold dilutions of 3G4 were prepared from an initial concentration of 13.33 nM. Dilutions were performed in 1% OVA or 10% nonheat-inactivated sera from cow, human, rat, or mouse. Plates were incubated for 1 h at 37 °C, and binding of 3G4 was detected as described previously (1). All ELISA experiments were performed at least three times, and representative figures are shown.

Anti-h β 2GP1 ELISA

The assay was performed as described above with the following modifications. h β 2GP1, nicked h β 2GP1, or recombinant h β 2GP1 were coated on 96-well Immunon 2HB microtiter plates overnight at a concentration of 10 μ g/ml. Plates were then blocked in 1% OVA for 1 h at room temperature. 3G4, ch3G4, or anti- β 2GP1 were diluted in 1% OVA to an initial concentration of 13.33 nM, and serial 2-fold dilutions were prepared. Plates were incubated for 1 h at 37 °C, and antibody binding was detected as described previously (1). All ELISA experiments were performed at least three times, and representative figures are shown.

Western Blot

Protein samples were heated to 95 °C for 5 min in nonreducing SDS sample buffer. The samples were then loaded onto a 4–15% Tris-HCl gradient SDS-polyacrylamide gel and separated using a Mini Protean II apparatus (Bio-Rad). Separated proteins were transferred to a polyvinylidene difluoride membrane and blocked overnight in 3% BSA (w/v). Membranes were probed with anti- β 2GP1, 3G4, or control mouse IgG diluted to 1 μ g/ml in 3% BSA, washed thoroughly, and incubated with

Anti-tumor mAb Requires β 2GP1 for Binding Endothelial Cells

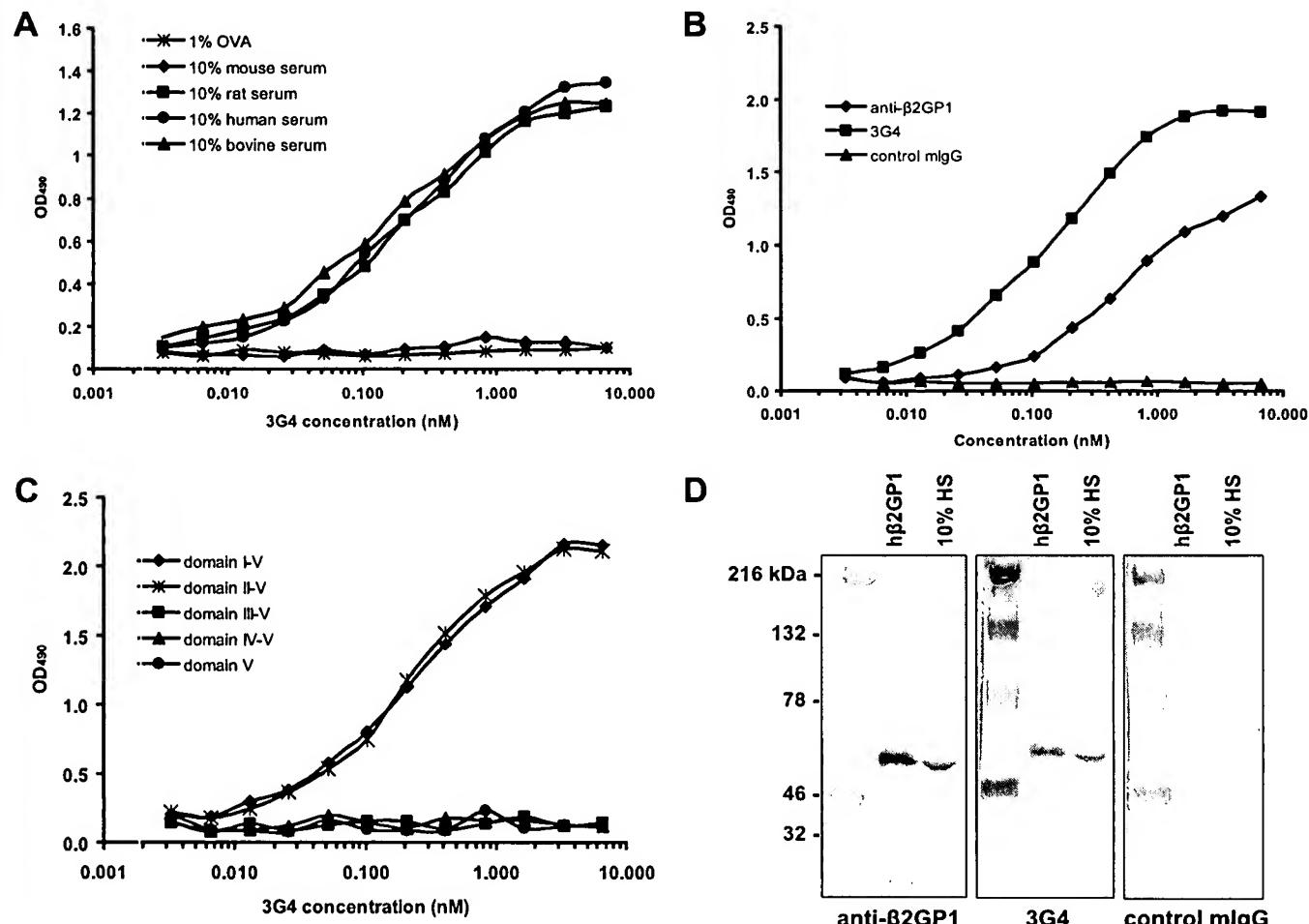


FIGURE 1. 3G4 binds plasma protein β 2GP1. *A*, microtiter plate was coated with PS and blocked in 1% OVA. Serial dilutions of 3G4 were performed in 1% OVA or 10% serum from the mammalian species indicated in the legend. *B*, microtiter plate was coated with β 2GP1 purified from human plasma and blocked in 1% OVA. Serial dilutions of a commercial mouse anti- β 2GP1, 3G4, and a control mlgG were performed in 1% OVA. *C*, wells of a microtiter plate were coated with recombinant full-length h β 2GP1 (domain I–V) or h β 2GP1 proteins missing domain I (II–V), domains I and II (III–V), domains I–III (IV–V), or domains I–IV (V). The plate was blocked in 1% OVA, and serial dilutions of 3G4 were performed in 1% OVA. *D*, purified h β 2GP1 and 10% human serum (10% HS) were run on an SDS-polyacrylamide gel and transferred to a membrane support. Protein was detected by immunoblot with anti- β 2GP1, 3G4, or control mlgG.

Downloaded from www.jbc.org by on April 9, 2007

peroxidase-labeled goat anti-mouse IgG. Finally, membranes were developed using an Opti-4CN substrate kit.

Induction and Detection of PS Exposure on Endothelial Cells

Detection with Antibodies—Adult bovine aortic endothelial (BAE) cells were maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. BAE cells were removed from subconfluent cultures by brief exposure to 0.25% trypsin, 0.02% EDTA, and 8-well chamber slides were seeded with 2×10^4 cells/well. Following overnight culture, cells were washed gently with PBS and treated with 200 μ M LPC to induce PS exposure. LPC treatment was performed in the presence of 3G4, ch3G4, or control IgG (all at 2 μ g/ml) for 30 min at 37 °C in either 10% FBS or 10% normal mouse serum. When LPC treatment was performed in 10% mouse serum, h β 2GP1 was added as a co-factor because 3G4/ch3G4 does not bind PS in mouse serum (see “Results”). Binding of antibodies to cells with exposed PS was determined by immunofluorescence staining. Cells were washed thoroughly in PBS, fixed in 4% paraformaldehyde (w/v), and incubated with a biotin-conjugated anti-mouse secondary antibody. Next,

cells were incubated with FITC-conjugated streptavidin (Jackson ImmunoResearch) to detect antibody binding. Finally, cells were permeabilized with 0.1% Triton X-100 in PBS and counterstained with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR) and 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes). Images were captured at a magnification of $\times 200$ using a Coolsnap digital camera (Photometrics, Tucson, AZ) mounted on a Nikon Eclipse E600 microscope and analyzed with MetaVue software (Universal Imaging Corp., Downingtown, PA).

Detection with Artificial Dimeric h β 2GP1 Constructs—Dimeric h β 2GP1 constructs apple4-C321S- β 2GP1 (h β 2GP1 dimer) and apple4-C321S- β 2GP1-W316S (mutant h β 2GP1 dimer) were generated as described previously (27). BAE cells were induced to exposed PS as described above. LPC treatment was performed in the presence of monomeric purified plasma h β 2GP1, h β 2GP1 dimer, or mutant h β 2GP1 dimer (all at 2 μ g/ml) for 30 min at 37 °C in 10% FBS. Cells were then washed thoroughly with PBS and fixed in 4% paraformaldehyde (w/v). Binding of the h β 2GP1 constructs to cells with exposed PS was detected with anti- β 2GP1, followed by staining with

appropriate immunofluorescent secondary detection reagents as described above.

Quantification of Antibody Binding to ABAE Cells

The area of antibody binding was determined using MetaVue image analysis software, which is able to quantify the number of illuminated pixels in an image. Images of FITC fluorescence were used to quantify antibody binding. Corresponding images of DAPI fluorescence were used to normalize the FITC images for the number of cells present in the field. A small FITC/DAPI ratio indicates a small antibody binding area, whereas a large FITC/DAPI ratio indicates a large binding area. The FITC/DAPI ratios were used to determine increases or decreases in

TABLE 1

Screening 3G4 for reactivity with serum proteins known to interact with phospholipids

Serum protein	3G4 reactivity ^a
Annexin V	—
β 2GP1	+
Factor XII	—
Kininogen	—
Oxidized low density lipoprotein	—
Protein C	—
Protein S	—
Prothrombin	—
tPA ^b	—

^a Equal amounts of indicated protein were coated on microtiter plates, blocked in 1% OVA, and incubated with 3G4 at 1 μ g/ml. Plates were washed thoroughly, and antibody binding was detected as described under "Materials and Methods." + indicates an A_{490} reading above 0.2, which is indicative of 3G4 binding. All experiments also included positive and negative control antibodies, which worked as expected.

^b tPA indicates tissue plasminogen activator.

antibody binding area relative to a basal amount of antibody binding under the selected conditions. Five images at $\times 200$ magnification were used for each analysis. Data are presented as average relative FITC/DAPI ratios with error bars representing the standard deviation.

RESULTS

3G4 Binds Serum Glycoprotein β 2GP1—3G4 bound to PS-coated microtiter plates when serial dilutions were performed in 10% bovine serum (Fig. 1A). In contrast, no 3G4 binding occurred when serial dilutions were performed in 1% OVA. This finding suggests that a factor present in bovine serum mediates the interaction between 3G4 and PS. To determine whether sera from other mammalian species can mediate the interaction between 3G4 and PS, serial dilutions of 3G4 were also performed in 10% mouse, rat, or human serum. 3G4 bound PS in the presence of rat and human serum but not in the presence of mouse serum (Fig. 1A). 3G4 also bound PS in the presence of hamster, ferret, guinea pig, rabbit, and monkey serum (data not shown). Therefore, with the exception of mouse, the serum protein epitope recognized by 3G4 seems to be conserved among mammalian species.

Because 3G4 was raised against EC induced to expose PS, it is possible that the reactivity of 3G4 is directed against serum proteins that bind PS. To test this possibility, microtiter plates were coated with a panel of known PS-binding proteins, and the reactivity of 3G4 against these proteins was determined. 3G4 bound only to β 2GP1 (Table 1 and Fig. 1B). To determine which domain of β 2GP1 is recognized by 3G4, five recombinant h β 2GP1 proteins were generated. These proteins lack various N-terminal domains because of serial truncations from the N terminus. Each protein was coated on a microtiter plate and incubated with a serial dilution of 3G4. Only proteins containing domain II of h β 2GP1 were detected by 3G4 (Fig. 1C). To determine whether β 2GP1 is the only serum protein recognized by 3G4, purified h β 2GP1 and 10% human serum were run on an SDS-polyacrylamide gel and transferred to a membrane support for immunoblot. 3G4 detected the 50-kDa purified h β 2GP1 and a single band of similar size in human serum (Fig. 1D). Importantly, the 3G4 immunoblot is virtually identical to a blot generated using an anti- β 2GP1 antibody. A control mIgG antibody did not detect any protein. Together, these data demonstrate that 3G4 binds β 2GP1, and binding is dependent upon domain II.

β 2GP1 Is Required for Binding of 3G4 to EC with Exposed PS—A live cell binding assay was developed to

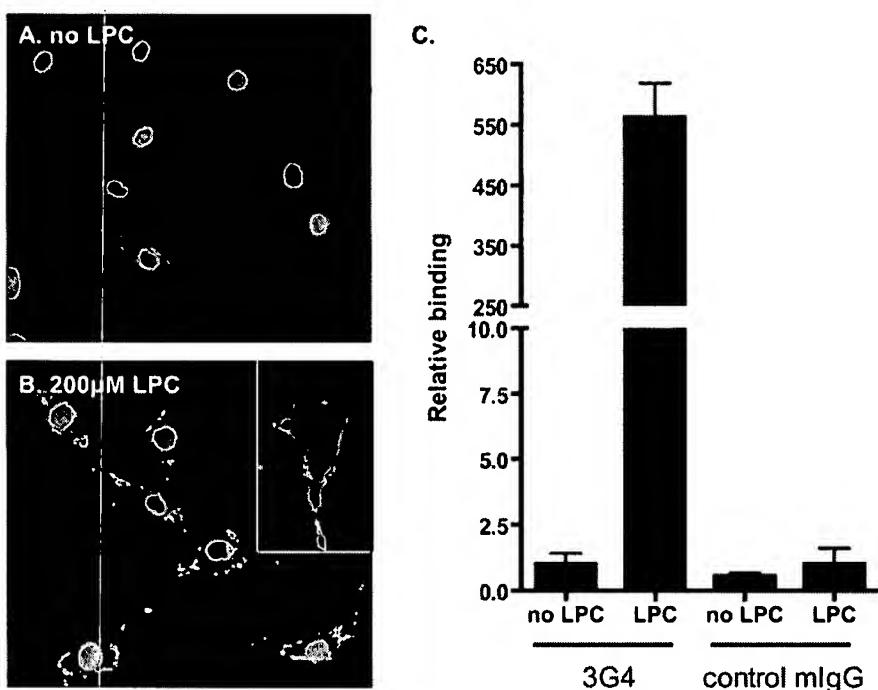


FIGURE 2. 3G4 binds to the surface of EC treated with lysophosphatidylcholine to induce PS exposure. ABAE cells were incubated with 3G4 or control mIgG in DMEM + 10% FBS in the presence or absence of 200 μ M LPC for 30 min. Cells were then washed, fixed, and stained with fluorescent markers to visualize binding of antibody to the cell surface. Non-LPC-treated (A) and LPC-treated (B) cells incubated with 3G4 are shown at $\times 200$ magnification (inset is at $\times 400$). The cytoskeleton appears red; nuclei appear blue, and 3G4 binding appears green. C, the pixel area of 3G4 or mIgG binding was quantified using MetaVue software. All values are relative to the binding of 3G4 to non-LPC-treated cells, which was arbitrarily set to 1.

Anti-tumor mAb Requires β 2GP1 for Binding Endothelial Cells

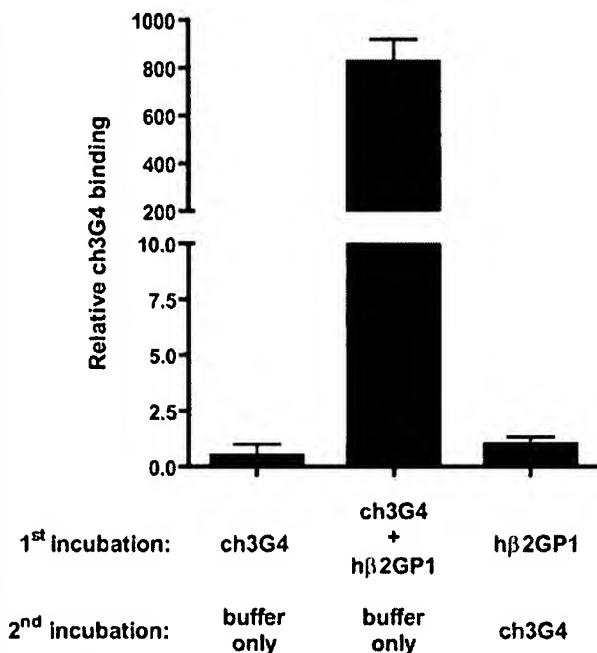


FIGURE 3. ch3G4 and β 2GP1 must be present simultaneously to bind EC with exposed PS. ABAE cells were incubated for 30 min with 200 μ M LPC in DMEM + 10% mouse serum, plus (i) ch3G4 only, (ii) ch3G4 + h β 2GP1, or (iii) h β 2GP1 only. Cells were then washed and incubated for 30 min in DMEM + 10% mouse serum, plus (i) nothing, (ii) nothing, or (iii) ch3G4, respectively. The cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. ch3G4 and h β 2GP1 were used at a concentration of 2 μ g/ml. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of ch3G4 under condition i, which was arbitrarily set to 1.

detect and measure 3G4 binding to EC membrane surfaces with exposed PS. When 3G4 was added to ABAE cell culture media under normal conditions, no binding to the cells was observed (Fig. 2A). However, when ABAE cells were incubated with 3G4 in the presence of the membrane-disrupting agent LPC, numerous pinpoints of 3G4 binding were readily detectable (Fig. 2B). LPC is known to induce temporary membrane distortions (28), which likely cause a loss of membrane asymmetry and exposure of PS. In this regard, similar results were obtained using the PS-binding protein annexin A5 (data not shown). LPC-treated ABAE cells were not stained by the membrane-impermeant dyes propidium iodide or DAPI (data not shown), indicating 3G4 and annexin A5 bound PS exposed on the cell surface following LPC treatment. The area of 3G4 binding increased more than 500-fold upon LPC treatment, whereas binding of a control mIgG remained undetectable (Fig. 2C).

To determine whether β 2GP1 is required for binding of 3G4 to EC with exposed PS, the live cell binding assay was performed in media containing 10% mouse serum instead of 10% FBS to prevent interference from bovine β 2GP1. For this experiment, a human chimeric 3G4 (ch3G4) was used to exclude nonspecific background caused by detection of murine IgG present in mouse serum. Similar to the results shown in Fig. 1, 3G4 did not bind LPC-treated EC in the presence of mouse serum (Fig. 3). In contrast, addition of purified h β 2GP1 to the cells supported widespread binding of ch3G4. Interestingly, when ABAE cells were incubated

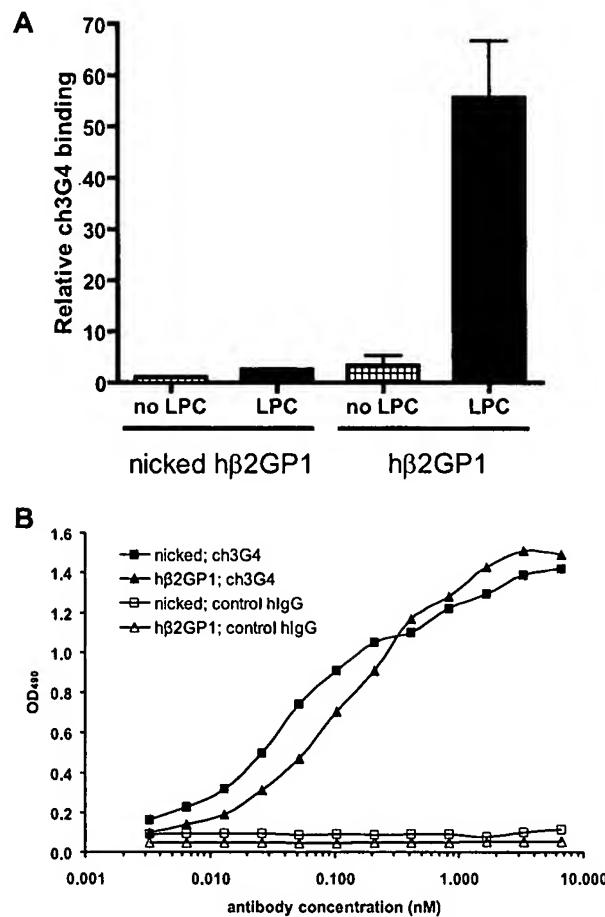


FIGURE 4. The lipid binding region of β 2GP1 is required to mediate binding of ch3G4 to EC with exposed PS. *A*, ABAE cells were incubated with ch3G4 plus (i) a non-lipid binding form of β 2GP1 (nicked h β 2GP1) or (ii) intact h β 2GP1. The incubations were performed in the presence or absence of 200 μ M LPC in DMEM + 10% mouse serum for 30 min. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. ch3G4, h β 2GP1, and nicked h β 2GP1 were used at a concentration of 2 μ g/ml. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of ch3G4 under condition (i), no LPC, which was arbitrarily set to 1. *B*, the wells of a microtiter plate were coated with h β 2GP1 or nicked h β 2GP1 and blocked in 1% OVA. Serial dilutions of ch3G4 or a control hIgG were performed in 1% OVA.

with h β 2GP1 in the presence of 10% mouse serum and LPC, washed thoroughly, and then incubated with ch3G4 to detect binding of h β 2GP1, very little ch3G4 binding was observed. This finding suggests that h β 2GP1 does not bind strongly to EC with exposed PS in the absence of ch3G4 and is consistent with reports that β 2GP1 has a low affinity for anionic phospholipid membrane surfaces under physiologic conditions (29, 30). Alternatively, excess mouse β 2GP1 present in the mouse serum could compete for binding of h β 2GP1, but this possibility is unlikely because similar results were obtained when the experiment was performed under serum-free conditions (data not shown). In all situations, ch3G4 binding was dependent upon LPC treatment, and no binding was detected using a control human IgG of irrelevant specificity. Together, these data show that ch3G4 and h β 2GP1 must be present simultaneously to bind ABAE cells with exposed PS, suggesting that cross-linking of β 2GP1 mole-

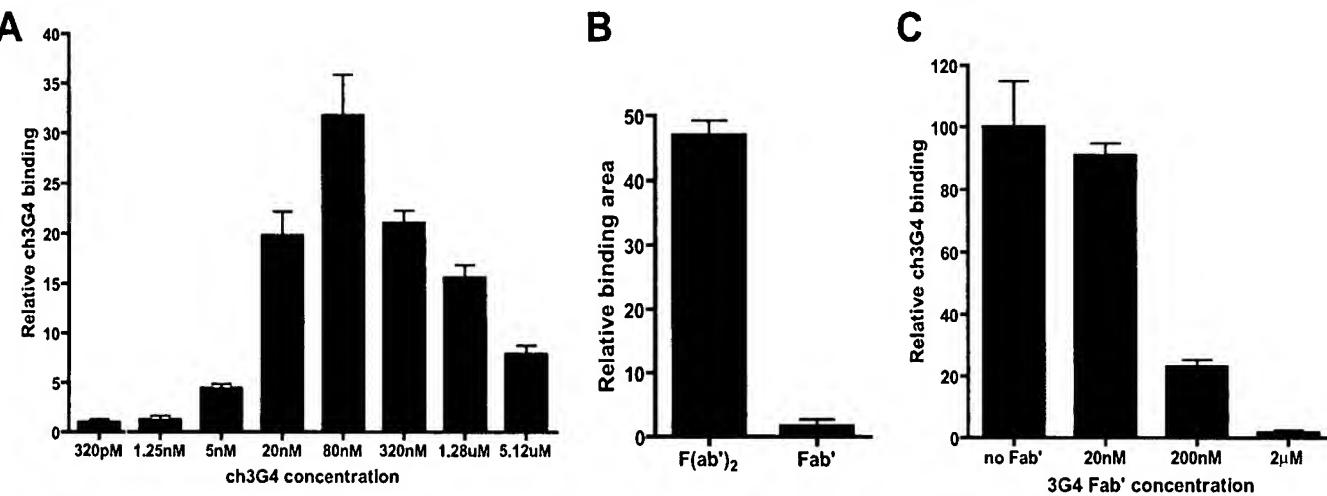


FIGURE 5. 3G4 divalency is required for β 2GP1-mediated binding to EC with exposed PS. *A*, ABAE cells were incubated for 30 min with 200 μ M LPC, 40 nM purified h β 2GP1, and increasing concentrations of ch3G4 in DMEM + 10% mouse serum. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of 320 pM ch3G4, which was arbitrarily set to 1. *B*, ABAE cells were incubated for 30 min with 20 nM 3G4 F(ab')₂ or 3G4 Fab' monomer in the presence 200 μ M LPC in DMEM + 10% FBS. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of the 3G4 fragments. The pixel area of antibody binding was quantified using MetaVue software. Values are relative to the binding of 3G4 in the absence of LPC (not shown), which was arbitrarily set to 1. *C*, ABAE cells were incubated for 30 min with 200 μ M LPC, 40 nM purified h β 2GP1, 20 nM ch3G4, and increasing concentrations of 3G4 Fab' monomer in DMEM + 10% mouse serum. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of ch3G4 without competing 3G4 Fab', which was arbitrarily set to 100.

cules by ch3G4 enhances the avidity of β 2GP1 for anionic phospholipid surfaces.

The Lipid Binding Region of β 2GP1 Is Required for β 2GP1-mediated Binding of ch3G4 to EC with Exposed PS—To determine whether the lipid binding region of β 2GP1 mediates binding of 3G4 to anionic phospholipids exposed on the surface of ABAE cells following LPC treatment, the live cell binding assay was performed using plasmin nicked h β 2GP1. Nicked h β 2GP1 does not bind anionic phospholipids because plasmin-mediated cleavage of domain V abrogates lipid binding (23, 31). When ABAE cells were incubated with ch3G4 and h β 2GP1 or nicked h β 2GP1 in the absence of LPC, no ch3G4 binding was detected (Fig. 4A). In the presence of LPC, h β 2GP1 mediated the binding of ch3G4 to ABAE cells with exposed PS, whereas nicked h β 2GP1 did not. The lack of binding in the live cell assay was not because of an inability of ch3G4 to bind nicked h β 2GP1, because ch3G4 bound nicked h β 2GP1 as strongly as h β 2GP1 when binding was assessed by ELISA (Fig. 4B). These findings demonstrate that the ch3G4-h β 2GP1 complex detects anionic phospholipids exposed on ABAE cells following LPC treatment through the lipid binding region of domain V.

Antibody Divalency Is Required for β 2GP1-mediated Binding of ch3G4 to EC with Exposed PS—The results presented above suggest 3G4 detects PS by enhancing the avidity of β 2GP1 for anionic phospholipids exposed on the surface of EC. To determine whether divalency is required for binding of 3G4- β 2GP1 complexes to anionic phospholipids, LPC-treated ABAE cells were incubated with purified h β 2GP1 and increasing concentrations of ch3G4. ch3G4 binding increased in a concentration-dependent manner until excess ch3G4 began to inhibit binding (Fig. 5A). The bell-shaped relationship between the concentration of ch3G4 and bind-

ing to EC with exposed PS suggests the formation of monomeric ch3G4- β 2GP1 complexes at very high antibody concentrations. The inability of these monomeric complexes to bind anionic membrane surfaces would explain the observed decrease in the amount of ch3G4- β 2GP1 complex bound to the LPC-treated ABAE cells. Therefore, the ability of 3G4 F(ab')₂ and 3G4 Fab' monomers to bind LPC-treated ABAE cells was determined. As expected, 3G4 F(ab')₂ bound to EC with exposed PS, but binding of 3G4 Fab' was negligible (Fig. 5B). No binding of 3G4 Fab' was detectable on ABAE cells even at a concentration of 2 μ M, which is 1000-fold above the concentration required to bind β 2GP1 coated on microtiter plates (data not shown). Finally, 3G4 Fab' inhibited ch3G4- β 2GP1 binding to LPC-treated ABAE cells in a concentration-dependent manner (Fig. 5C), whereas a control Fab' of irrelevant specificity did not (data not shown). The ability of 3G4 Fab' to inhibit the binding of ch3G4 indicates that 3G4 Fab' binds β 2GP1 and that monomeric 3G4 Fab'- β 2GP1 complexes do not bind EC with exposed PS.

Artificial Dimeric β 2GP1 Construct Binds to EC with Exposed PS—The findings described above indicate that binding of 3G4- β 2GP1 complexes to EC with exposed PS is dependent on the increased avidity of β 2GP1 for anionic phospholipids when cross-linked by 3G4. To verify this, an artificially generated h β 2GP1 dimer (27) was used to determine whether dimeric β 2GP1 can bind EC with exposed PS in the absence of 3G4. ABAE cells were treated with LPC to induce PS exposure and incubated with purified h β 2GP1, h β 2GP1 dimer, or a mutant h β 2GP1 dimer containing a disrupted lipid binding domain. The cells were then washed and β 2GP1 binding was determined with anti- β 2GP1. As expected, binding of the monomeric h β 2GP1 to LPC-treated ABAE cells was negligible (Fig. 6A). In contrast, the h β 2GP1

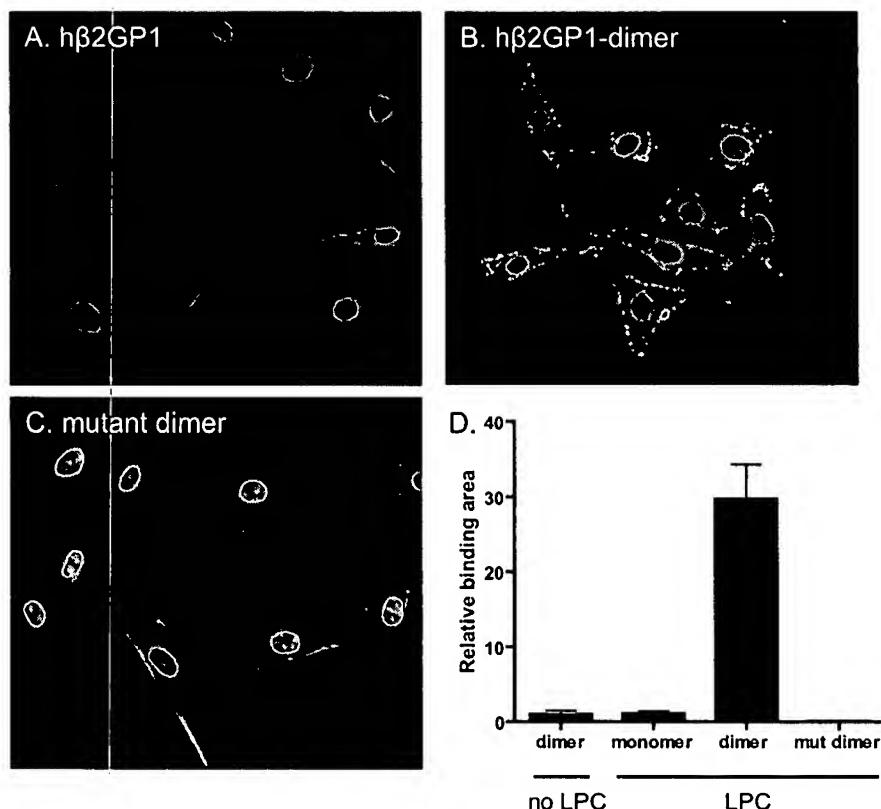
Anti-tumor mAb Requires β 2GP1 for Binding Endothelial Cells

FIGURE 6. An artificial dimeric β 2GP1 construct binds EC with exposed PS. ABAE cells were incubated for 30 min with 200 μ M LPC in DMEM + 10% FBS plus purified h β 2GP1 monomer (A), h β 2GP1 dimer (B), or a mutant h β 2GP1 dimer unable to bind lipid (C). Cells were then washed, fixed, and incubated with anti- β 2GP1 to detect h β 2GP1 monomers and dimers. Finally, cells were stained with fluorescent markers; the cytoskeleton appears red; nuclei appear blue, and h β 2GP1-monomers and dimers appear green. D, the binding area of h β 2GP1 monomers and dimers was quantified using MetaVue software. All values are relative to the binding of h β 2GP1 dimers to non-LPC treated cells, which was arbitrarily set to 1.

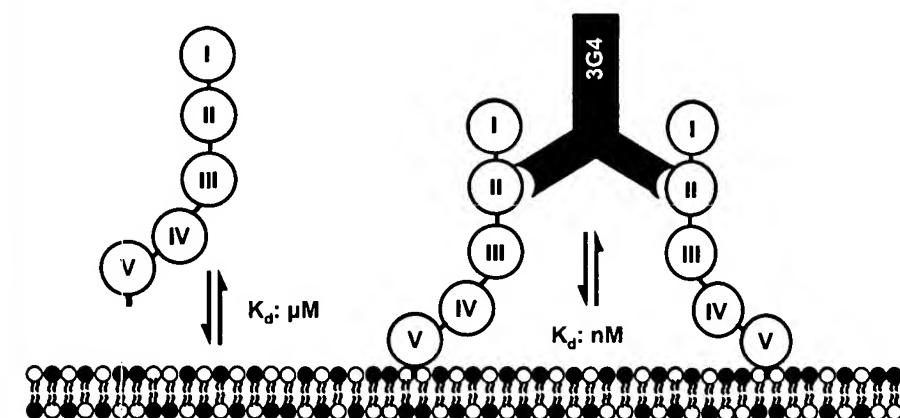


FIGURE 7. Model of 3G4/ β 2GP1 binding to endothelial cell membrane surfaces with exposed anionic phospholipids. Single molecules of β 2GP1 have low affinity for anionic phospholipid membrane surfaces with a dissociation constant of \sim 4 μ M (32). Binding of 3G4 to two molecules of β 2GP1 (via domain II of β 2GP1) strongly enhances the avidity of the 3G4- β 2GP1 complex for membrane surfaces with exposed anionic phospholipids (depicted as open circles in the lipid bilayer). The avidity of the 3G4- β 2GP1 complex for anionic phospholipids may increase 1000-fold or more into the low nanomolar range (29, 30).

dimer bound strongly to LPC-treated cells (Fig. 6B) but not to non-LPC-treated cells (Fig. 6D). Finally, binding of the h β 2GP1 dimer was dependent upon a functional lipid binding domain, because the mutant dimer did not bind (Fig. 6C). These data indicate 3G4 binding to EC with exposed PS is

primarily because of the enhanced avidity of dimeric β 2GP1 complexes for PS.

DISCUSSION

The objective of this study was to characterize the interaction between the tumor vascular targeting antibody, 3G4, and its anionic phospholipid target, PS. We demonstrated that the interaction between 3G4 and PS is serum-dependent, and we identified β 2GP1 as the serum co-factor required to mediate the interaction between 3G4 and PS. 3G4 was originally generated by immunizing mice with murine EC treated with H_2O_2 to induce PS exposure (1). These cells were grown in FBS-containing media where bovine β 2GP1 was likely associated with surface-exposed PS, leading to production of the 3G4 antibody. This is not surprising because many "anti-phospholipid antibodies" have been shown to require serum co-factors for lipid binding (32, 33).

Similar to the ELISA-based findings, which demonstrated that binding of 3G4 to PS-coated plates is β 2GP1-dependent, the live cell binding assay showed that β 2GP1 is required for binding of 3G4 to EC with exposed PS. Interestingly, binding of h β 2GP1 to EC with exposed PS was negligible in the absence of ch3G4. This finding is consistent with reports that β 2GP1 has low affinity for anionic phospholipid membranes under physiological conditions but increases \sim 1000-fold upon cross-linking with anti- β 2GPI antibodies (29, 30). This suggests that 3G4 also mediates the formation of divalent-multivalent β 2GP1 complexes. Importantly, high concentrations of 3G4 inhibited the binding of 3G4- β 2GP1 complexes to EC with exposed PS. The bell-shaped relationship between antibody concentration and cell bind-

ing raises the possibility that at high antibody concentrations an increasing fraction of monovalent antigen-antibody complexes effectively decreases the propensity of the multivalent complexes to bind EC with exposed PS (34). Indeed, 3G4 Fab' fragments incubated with cells in the presence of β 2GP1 failed

Anti-tumor mAb Requires β 2GP1 for Binding Endothelial Cells

to bind to anionic phospholipid membrane surfaces. Further evidence in support of the notion that bivalent β 2GP1 is required for EC binding was obtained from experiments that showed that artificially generated dimeric β 2GP1 molecules bound EC in the absence of antibody. Taken together, these findings support the hypothesis that 3G4 effectively increases the avidity of β 2GP1 for anionic phospholipid surfaces through the formation of multimeric β 2GP1-antibody complexes (35, 36).

We have shown previously that 3G4 has potent anti-tumor effects in mice (1, 2, 18). 3G4 used in these earlier studies was isolated from media containing bovine serum. In contrast to the 3G4 isolated from serum-free media used in the studies presented here, 3G4 isolated from serum-containing media binds PS-coated microtiter plates in the absence of exogenously added β 2GP1 because the antibody co-purifies with bovine β 2GP1. Indeed, fast protein liquid chromatography analysis of 3G4 isolated from serum-containing media indicated that ~10% of 3G4 was complexed with bovine β 2GP1.³ Because bovine β 2GP1 mediates binding of 3G4 to PS in the presence of mouse plasma, the presence of the bovine protein likely contributed to the anti-tumor effects of 3G4 observed in our previous studies. As expected, good tumor localization is observed in mice when 3G4 is supplemented with excess h β 2GP1.⁴ Moreover, specific localization of 3G4 to tumor vascular endothelium is observed in rats bearing syngeneic AT1 prostate tumors in the absence of exogenous bovine or human β 2GP1, because 3G4 binds the endogenous rat β 2GP1 (37).

In conclusion, the identification of β 2GP1 as a critical co-factor for the interaction between 3G4 and PS enhances our understanding of this unique tumor vascular targeting agent (1, 2, 18). Our findings show 3G4 binds EC with exposed PS by enhancing the avidity of β 2GP1 for anionic phospholipid surfaces through the formation of multimeric 3G4- β 2GP1 complexes (Fig. 7). This interaction likely enables 3G4 to target tumor vascular endothelial cells with exposed PS *in vivo*.

REFERENCES

1. Ran, S., He, J., Huang, X., Soares, M., Scothorn, D., and Thorpe, P. E. (2005) *Clin. Cancer Res.* **11**, 1551–1562
2. Huang, X., Bennett, M., and Thorpe, P. E. (2005) *Cancer Res.* **65**, 4408–4416
3. Daleke, D. L. (2003) *J. Lipid Res.* **44**, 233–242
4. Devaux, P. F. (1992) *Annu. Rev. Biophys. Biomol. Struct.* **21**, 417–439
5. Bitbol, M., Fellmann, P., Zachowski, A., and Devaux, P. F. (1987) *Biochim. Biophys. Acta* **904**, 268–282
6. Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., and Sims, P. J. (1997) *J. Biol. Chem.* **272**, 18240–18244
7. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995) *J. Exp. Med.* **182**, 1545–1556
8. Dillon, S. R., Mancini, M., Rosen, A., and Schlissel, M. S. (2000) *J. Immunol.* **164**, 1322–1332
9. Boyle, E. M., Jr., Pohlman, T. H., Cornejo, C. J., and Verrier, E. D. (1996) *Ann. Thorac. Surg.* **62**, 1868–1875
10. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J. (1991) *Cancer Res.* **51**, 3062–3066
11. Shaughnessy, S. G., Buchanan, M. R., Turple, S., Richardson, M., and Orr, F. W. (1989) *Am. J. Pathol.* **134**, 787–796
12. Orr, F. W., Wang, H. H., Lafreniere, R. M., Scherbarth, S., and Nance, D. M. (2000) *J. Pathol.* **190**, 310–329
13. Soares, F. A., Shaughnessy, S. G., MacLarkey, W. R., and Orr, F. W. (1994) *Lab. Investig.* **71**, 480–489
14. Zulueta, J. J., Yu, F. S., Hertig, I. A., Thannickal, V. J., and Hassoun, P. M. (1995) *Am. J. Respir. Cell Mol. Biol.* **12**, 41–49
15. Ran, S., Downes, A., and Thorpe, P. E. (2002) *Cancer Res.* **62**, 6132–6140
16. Ran, S., and Thorpe, P. E. (2002) *Int. J. Radiat. Oncol. Biol. Phys.* **54**, 1479–1484
17. Ran, S., Gao, B., Duffy, S., Watkins, L., Rote, N., and Thorpe, P. E. (1998) *Cancer Res.* **58**, 4646–4653
18. Beck, A. W., Luster, T. A., Miller, A. F., Holloway, S. E., Conner, C. R., Barnett, C. C., Thorpe, P. E., Fleming, J. B., and Brekken, R. A. (2005) *Int. J. Cancer* **118**, 2639–2643
19. Steinbacher, A., Estaller, C., Weiss, E. H., Sim, R. B., and Day, A. J. (1991) *Biochem. J.* **277**, 387–391
20. Steinbacher, A., Barlow, P. N., Willis, A. C., Kertesz, Z., Campbell, I. D., Sim, R. B., and Norman, D. G. (1992) *FEBS Lett.* **313**, 193–197
21. Sheng, Y., Sali, A., Herzog, H., Lahnstein, J., and Krilis, S. A. (1996) *J. Immunol.* **157**, 3744–3751
22. Mehdi, H., Naqvi, A., and Kamboh, M. I. (2000) *Eur. J. Biochem.* **267**, 1770–1776
23. Hunt, J., and Krilis, S. (1994) *J. Immunol.* **152**, 653–659
24. Ran, S., Huang, X., Downes, A., and Thorpe, P. E. (2003) *Neoplasia (Bratisl.)* **5**, 297–307
25. Polz, E., Wurm, H., and Kostner, G. M. (1980) *Int. J. Biochem.* **11**, 265–270
26. Wurm, H. (1984) *Int. J. Biochem.* **16**, 511–515
27. Lutters, B. C., Meijers, J. C., Derkens, R. H., Arnout, J., and de Groot, P. G. (2001) *J. Biol. Chem.* **276**, 3060–3067
28. Kogure, K., Nakashima, S., Tsuchie, A., Tokumura, A., and Fukuzawa, K. (2003) *Chem. Phys. Lipids* **126**, 29–38
29. Willems, G. M., Janssen, M. P., Pelsers, M. M., Comfurius, P., Galli, M., Zwaal, R. F., and Bevers, E. M. (1996) *Biochemistry* **35**, 13833–13842
30. Bevers, E. M., Janssen, M. P., Comfurius, P., Balasubramanian, K., Schroit, A. J., Zwaal, R. F., and Willems, G. M. (2005) *Biochem. J.* **386**, 271–279
31. Hunt, J. E., Simpson, R. J., and Krilis, S. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2141–2145
32. Bevers, E. M., Zwaal, R. F., and Willems, G. M. (2004) *Clin. Immunol.* **112**, 150–160
33. Roubey, R. A., Eisenberg, R. A., Harper, M. F., and Winfield, J. B. (1995) *J. Immunol.* **154**, 954–960
34. Jankowski, M., Vreys, I., Wittevrongel, C., Boon, D., Vermylen, J., Hoylaerts, M. F., and Arnout, J. (2003) *Blood* **101**, 157–162
35. Galazka, M., Keil, L. B., Kohles, J. D., Li, J., Kelty, S. P., Petersheim, M., and DeBari, V. A. (1998) *Thromb. Res.* **90**, 131–137
36. Gushiken, F. C., Le, A., Arnett, F. C., and Thiagarajan, P. (2002) *Thromb. Res.* **108**, 175–180
37. Jennewein, M., Lavine, N., He, J., Kodibagkar, V., Kulkarni, P. V., Selcuklu, S., Lewis, M., Zhao, D., Tysyanov, E., Antich, P. P., Hermann, A., O'Kelly, S., Qaim, S. M., Roesch, F., Mason, R. P., and Thorpe, P. E. (2005) *Proc. Am. Assoc. Cancer Res.* **46**, 5261

³ J. He and P. E. Thorpe, unpublished observations.⁴ T. A. Luster, J. He, X. Huang, and P. E. Thorpe, unpublished observations.